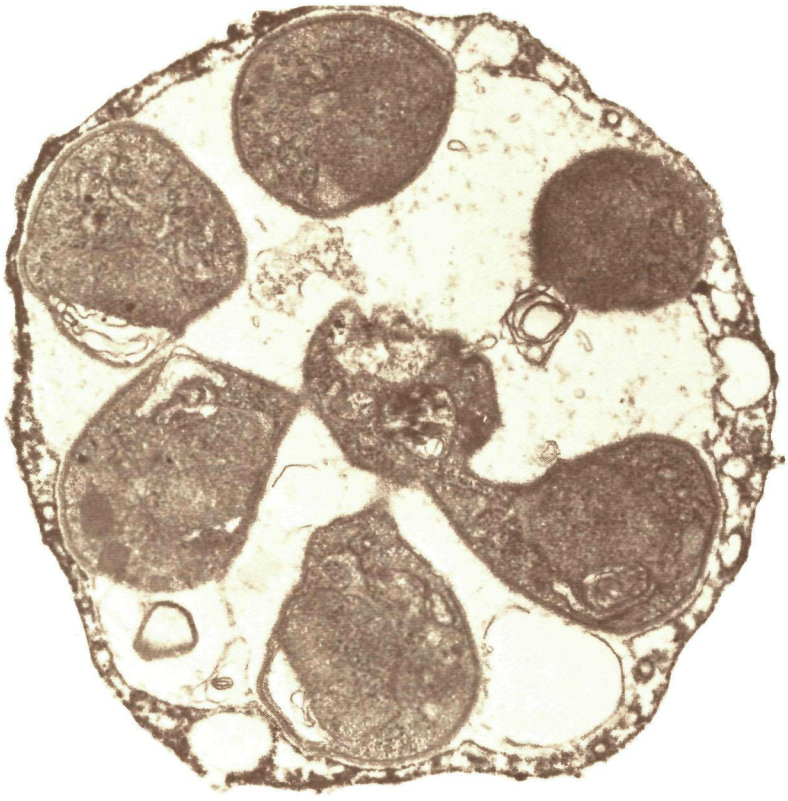
RELEASE OF ANTIGENS AND IMMUNOGENICITY VERSUS THE HOST RESPONSE DURING INFECTION WITH A RODENT MALARIA PARASITE



L. G. POELS

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Promotor: Prof. Dr. C.R. Jerusalem

This thesis has been prepared in the Laboratory of Cytology and Histology, Katholieke Universiteit, Nijmegen, The Netherlands.

This study was financially supported by the World Health Organization, and by the International Collaborative Research Programme on Malarial Immunity and Immunopathology (ICRP/MII) supported by the Dutch Government.

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PROEFSCHRIFT

ter verkrijging van de graad van Doctor in de Geneeskunde aan de Katholieke Universiteit te Nijmegen op gezag van de Rector Magnificus Prof. Dr. A.J.H. Vendrik, volgens besluit van het College van Decanen in het openbaar te verdedigen op vrijdag 2 december 1977 des namiddags te 2.00 uur precies

door

Lambert Gerard Poels
geboren te Meerlo

1977

druk: Krips Repro B.V. Meppel

To ALL who have contributed

in completing this thesis

CONTENTS

CHAPTER 1 GENERAL INTRODUCTION

The present state of malaria control in many areas of the world gives no cause for complacency. The progress of malaria eradication programme has been slow in the past few years and has even suffered significant setbacks in some large countries such as India and Pakistan. Data reported by the World Health Organization (1975) show that end 1974 nearly 800 million people living in originally malarious areas have been freed from the risk of malaria, and that an additional 773 million are being protected by active programmes. On the other hand, 363 million live in heavily endemic areas where no specific antimalaria measures are applied. On the continent of Africa alone, 96 million cases of malaria occur annually; one million, usually those involving young children, end in death (WHO 1974; 1975).

The increasing emergence of drug-resistant strains of human plasmodia and of insecticide-resistant strains of mosquitoes, as well as difficulties of social-economic order can cause resurgence of malaria.

One of the most dramatic examples of resurgence of malaria (Bruce-Chwatt 1974) is that of Sri-Lanka with a population of about 12 million. In 1963 the number of recorded cases fell to 18, and a year later the total coverage by residual DDT spraying was interrupted. But during the period 1968-1970 some 1.5 million cases were reported. Renewed attack measures reduced the number of cases to 150.000 in 1971. In 1976, however, the number of cases was estimated to about 450.000. The increasing insecticide resistance of the vectors and the shortage of funds after the recent energy crisis seems to endanger again the eradication programme in Sri-Lanka severly (Dr. T. Ponnudurai and Dr. R. Bernard, Sri Lanka, personal communication).

These figures amply illustrate the magnitude of morbidity and mortality caused directly by malaria. In spite of great achievements of the global malaria eradication programme, a large reservoir of endemic malaria remains over most of the tropics. Although Europe has become largely free of endemic malaria, a break-through in Turkey has been reported recently by Dr. S. Goriup (personal communication). Europe has become a major receiving area for imported malaria cases and the relevant case-fatality rates are higher than in areas with high malaria endemicity, owing to low immunity in the majority of cases and missed or delayed diagnosis (WHO 1974). Although considerable progress in malaria eradication has been made in the past years, it is clear that malaria will be with us for quite some time and that alternative approaches to malaria control will be necessary (Lepes 1974).

In the search for new ways of controlling or eradicating malaria, research on the immune response following malarial infection are of great importance for the development of immunizing agents. Although no animal model can provide the final verdict on immunological methods of protection against human malaria, rodent malaria infections are useful for detailed analysis of the immune response of the host to the parasites, the development of acquired protective immunity, and the immunopathological lesion associated with malaria.
Extensive experimental studies of immunity to malaria have been published and the readers are referred to the excellent reviews by Brown (1969), McGee (1970), Garnham (1970), Zuckerman (1970) and Manuel and Behin (1974), and a report of a WHO Scientific Group (1975).

Although the final goal of malaria research might be the development of a malaria vaccine, an alternative approach was thought to be the use of "immunogenic" (immune or informational) RNA which could possibly be instrumental in evoking a rapid antimalaria response. The basis for this type of research was provided originally by experiments of Fishman and Adler (1963) and Mitsuhashi and Saito (1962) showing that cellular immunity was acquired when the ribosomal fraction of monocytes from rats immunized with Salmonella enteritidis was transferred to monocytes of normal animals. The transfer of immunity to a nonimmune recipient by "immune" ribonucleoprotein particles (RNP) or by RNA was considered to be of great practical importance. First, an immunologically defective host could be effectively corrected with regard to transplantation or tumor immunity; and second, the RNA did apparently not have to be strain or species specific (Paque and Dray 1970; Rigby 1969). A number of articles dealing with the conversion of nonimmune to immune immunocompetent cells, in vivo and in vitro, by "immune" RNP or RNA have been published. The donor "immune" RNA appeared to induce the synthesis of specific antibodies of donor light and heavy chain allotype (Bell and Dray 1971, 1972; 1973), homograft immunity (Likhitte, Sabbadini and Sehon 1973), tumor immunity (Deckers, Ramming and Pilch 1973) and autoimmunity (Nagy 1970). For a broad review of this field the readers are referred to a special volume (207, 1973) of the annals of the New York Academy of Sciences on "a conference on RNA in the immune response." The model of transfer of immunological information in vivo and in vitro is questionable. The extraneous RNA might function in three distinct ways.

(1). Firstly, it can act as "immune" RNA (IRNA) which transfers information pertaining to both the allotype and the antibody specificity of the immunoglobulin product: the IRNA functions in the additional role of template for RNA replicase and for reverse DNA transcriptase. However, the results of Jacherts (1967, 1968) were not completely reproducible by others (Bishop, Weiss and Hoffmann 1973), and a considerable variation occurred in the proportion of converted cells; and only some of the RNA batches tested were able to induce the synthesis of donor allotype antibodies.

(2). The second mechanism involves the activity of RNA-antigen complexes considered to function as a sort of superantigen (Askonas and Rhodes 1965; Gottlieb, Schwartz, Kudva and Waldman 1973). The precise role of such "superantigens" remains to be defined.

(3). Thirdly, there is clear an convincing evidence that RNA, and some of its precursors and synthetic analogues may act as signal complifiers in the antibody response. This adjuvant-like action appears to operate via stimulation of cyclic AMP. This stimulatory effect of RNA may also partly operate in the second mechanism mentioned above (Braun 1973). Normal "nonimmune" RNA obtained from spleens, is reported to inhibit immune responses (Morini, Londner, Font and Rabasa 1969; Londner, Morini, Armelio, Font and Rabasa 1972).

The evidence on the precise role of RNA in the induction of immune response appears extremely controversial (Theodorescu 1973), or at least of complex nature.

Nevertheless, in view of the above discussion it was thought that immune RNP and/or RNA particles obtained from lymphatic organs from malaria infected or immune mice might be

able to transfer immunity to noninfected normal mice.

Biological experiments with RNA and polysomes require methods for the isolation of original polysomal structures and the harvesting of adequate amounts of biologically active material. Therefore the formation of polyribosomes in spleens was studied in normal and experimental mice (Poels 1976, 1977 chapters 2/3).Transfer of splenic RNP particles obtained from mice infected with or immunized to Plasmodium berghei to normal recipients subsequently challenged did not affect the parasitemia (Jerusalem, Weiss and Poels 1971), but the number of animals succumbing in the period of the first peak mortality was either enhanced or reduced compared to controls, depending on the size of the inoculum. Protective immunity, however, could never be transferred by RNP or RNA fractions. Theoretically, all three mentioned mechanisms proposed for the actions of IRNA might have been involved. Spleens of recipients of IRNA showed stimulated germinal centers, however, the differences with controls were marginal. Numbers of experiments with splenic IRNA obtained either from infected or immune mice, or from mice stimulated with heterologous erythrocytes, were completely unsuccesful in inducing any more precisely defined specific response in vitro as well as in vivo. The negative results were independent of the purity of RNA fractions, doses of RNA, route of inoculation, or method of incubation with lymphocytes and timing of harvesting the RNA during the immunization schedule. The inability to repeat RNA-induction experiments reported in the literature, the personal communication of Dr. Dray that only some RNA preparations of

forty batches tested in their hands were effective inducers of donor allotype antibody synthesis, and the limited understanding of the immune response of the host to the parasite made it imperative to postpone the investigations on the role of IRNA until the host immune response to Plasmodium berghei infection in mice had been characterized more precisely.

The study on immune responses in mice to the blood born Plasmodium berghei parasite may not cover the complexity of immune reaction in human malaria, as only the erythrocytic cycle is reflected in the mouse model, and it does not express chronicity (Brown, Jarra and Hills 1976; Brown personal communication). The practical relevance of the variety of information obtained in laboratory experiments (chapter 4-6) neccesitates therefore the conformation in human malaria. In addition, the malaria infection per se offers an excellent although complicated, model for the study on the diversity of immune response of the host to a continually propagating parasite. First, Plasmodium berghei infection in mice severely disturbs the immune responsiveness (Poels and van Niekerk 1977, chapter 4), thus providing a certain model of immunodeficiency. Secondly, in rodent malaria the mutual interference of various types of immune responses not leading a priori to protective immunity can be investigated. Thirdly, the infection provides a model for studying the immunopathology (Poels, van Niekerk, Pennings, Agterberg and van Elven 1977, chapter 6). Fourthly, the model promises a differentiation between "protective and nonprotective" antigens of malaria parasites and the immune response to them.

The aim of these studies is considered to form the basis of a rationale for developing a malaria vaccine that excludes the risk of establishing immune reactions which may interfere with the protective immune response, and that does neither give rise to immunopathological lesions during vaccination, nor subsequent to challenge.

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CHAPTER 2 POLYRIBOSOME PROFILES AND RIBONUCLEASE ACTIVITY IN SPLEENS OF NORMAL AND ANAEMIC MICE

Reprinted from

Biochimica et Biophysica Acta, 432 (1976) 245—253

BBA 98581

POLYRIBOSOME PROFILES AND RIBONUCLEASE ACTIVITY IN SPLEENS OF NORMAL AND ANAEMIC MICE

LAMBERT G POELS

Department of Cyto Histology, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen (The Netherlands)

(Received September 11th, 1975)

Summary

The extent to which the polyribosome content in splenic extracts is affected by the level of endogenous ribonuclease activity was investigated. Ribonuclease activity, free and inhibited, was determined under ionic conditions optimal for preservation of polyribosomes. The results show that ribonuclease activity in control spleens varies mainly with the amount of ribonuclease inhibitor, indicating that free ribonuclease activity is stable. In rapidly proliferating splenic tissue the enzymatic activity is regulated by changes in both the amount of inhibitor and enzyme available. The ribonuclease versus ribonuclease-inhibitor balance was found to parallel the proportion of polyribosomes in a polysome profile.

Introduction

Biological experiments with RNA and polysomes require methods for the isolation of original polysomal structures and the harvesting of adequate amounts of biologically active material [1]. Changes in polysomal profiles, particularly their loss, may be caused by endogenous ribonuclease in the spleen [2—8]. Preservation of splenic polysomes was effected with the aid of a natural ribonuclease inhibitor [5], isolated from rat liver, although complete inhibition was not always possible [6]. Variations in splenic ribonuclease activity have been observed mainly after antigenic stimulation. Conflicting results [7,9] however, may be due to variations in the change of cell populations upon antigenic stimuli, i.e. either lymphocyte transformation or plasma cell formation [2,10]. The transformation of lymphocytes by phytohaemagglutinin was shown to be accompanied by a rise in ribonuclease inhibitor, the amount of which is high in lymphoblast and plasma cells [11,12]. The latter have an increased proportion of polyribosomes [2]. Less attention has been paid to the

erythropoietic activity of the spleen Massive proliferation of erythroblasts, which also exhibit large numbers of polysomes, can be induced by progressive anaemia following administration of phenylhydrazine or results from infection with the malarial parasite *Plasmodium berghei* [13]. In addition, the malarial parasite acts primarily as an antigen, stimulating the cells of the white pulp. This indicates that the yield of original polysomal material may not only depend upon the method of isolation, but also on the actual cellular composition of the spleen and the physical condition of the cell itself Therefore, we investigated the extent to which the polyribosomal content of splenic extracts is affected by the level of endogenous ribonuclease activity and its inhibitor using standardized experimental conditions for massive proliferation of mainly erythropoietic tissue. The ionic conditions were optimal for the preservation of polysomes, and the presence of Mg^{2+} in the medium is known to be inhibitory for ribonuclease and to stimulate the formation of the ribonuclease inhibitor complex [14].

Materials and Methods

Animals Specific pathogen-free Swiss mice, approx. 8 weeks old were used in these studies. They were maintained on laboratory standard diet (Hope Farms Ltd) and water ad libitum.

Malarial infection Swiss mice were inoculated intraperitoneally with 10^5 parasitized (*Plasmodium berghei*) red blood cells During the course of infection the haemoglobin content gradually decreased from 15 g% to 4 g%, whereas the proportion of polychromatophilic erythrocytes increased to approx. 45%. The spleen weight could increase up to about 10 times [13].

Phenylhydrazine treatment Mice were injected subcutaneously with 0 2 ml of a 1% neutralized phenylhydrazine solution on several consecutive days Animals which responded rapidly exhibited about 20% polychromatophilic erythrocytes on day 4—5, and spleens weighed approx 600 mg.

Preparation of splenic extracts The spleens were excised and placed in cold homogenising buffer, consisting of 50 mM Tris/HCl, 10 mM $MgCl_2$ and 25 mM KCl, pH 7.6. The spleens were homogenised manually in a teflon-glass homogeniser by five strokes. Cellular debris was removed by centrifugation for 10 min at 7000 rev./min (10 000 × *g*) in a Sorvall RC-2B centrifuge. The resulting supernatant (S-10 fraction) was used for ribonuclease assay and for analysis of polyribosomal content. For some experiments the S-10 fraction was layered on top of 3 ml 25% sucrose in homogenisation buffer. The polyribosomes were collected by centrifugation for 1 h at 65 000 rev./min at 2°C in the SW-65 rotor of an L2-65 ultracentrifuge. The ribosomal pellets of 3 tubes were resuspended in 1 ml of buffer, and the suspension clarified by centrifugation for 5 min at 6000 rev./min at 20°C in a Sorvall centrifuge. The supernatant (SP-6 fraction) was used for ribonuclease assay and analysis of polyribosomal content.

Sucrose density gradients An 0.25 ml sample of the S-10 fraction was layered on top of an 11.5 ml 8—40% linear sucrose gradient in homogenisation buffer. The gradients were centrifuged for 90 min at 40 000 rev./min in the SW-40 rotor of a Spinco ultracentrifuge Model L2-65B. The centrifuged gradi-

ents were analysed by passing through the flow cell of a Zeiss recording spectrophotometer PMQ 11 with the wavelength set at 260 nm. The proportion of polyribosomes in the total profile was determined planimetrically.

Ribonuclease assay Ribonuclease activity was measured by a modification of the method of Shortman [15]. The reaction mixture used consisted of 0 25 ml of S-10 fraction and 0.20 ml of 1% yeast RNA in homogenisation buffer, to which was added either 0.10 ml buffer or 0.10 ml *p*-chloromercurbenzoate ($ClHgBzO^-$) to a final concentration of 1.8 mM. This concentration was found sufficient to release all ribonuclease activity, when the protein concentration in the incubation mixture was kept under 0.5 mg/ml. The assay tubes were incubated for 30 min at either 37 or 0°C The reaction was stopped by placing the tubes in ice for 1 min. Subsequently, 1 ml 0 1 M HCl in 76% ethanol (—20°C) was added, and the tubes stored at —20°C for 2.5 h. The samples were then centrifuged at 3000 rev /min at 4°C An aliquot of the clear supernatant was diluted with distilled water and the absorbance at 260 nm determined. The protein content of the sediment was determined by the method of Lowry et al [16]. The specific ribonuclease activity was expressed in A_{260} units per mg protein.

Ribonuclease inhibitor Rat liver supernatant was used as a source of ribonuclease inhibitors. Male Wistar rats were starved for 24 h before decapitation The liver was quickly excised, washed and homogenised with 2 ml homogenisation buffer per g wet wt. The 100 000 × *g* supernatant was prepared as described by Gribnau et al. [17]. The supernatant was freshly prepared on the day of use In the same way, a 100 000 × *g* supernatant of spleens of Swiss mice was prepared. The supernatants of normal Swiss mice and of mice 14 days after malaria infection were used in the experiments.

Chemicals Yeast RNA, grade VI was obtained from Sigma Chemical Co. and repurified by six consecutive precipitations with 1% NaCl in ethanol at —20°C. The final precipitate was dried, dissolved in homogenisation buffer, and adjusted to a 1% solution (1 μg/ml A_{260} = 0.020) The ammonium salt of aurintricarboxylic acid (Merck) was dissolved in the buffer. The final concentrations in the assay mixture were 1 mM and 0 1 mM. Triton X-100 (Sigma) in concentrations from 0.1 to 1 0%, and sodium deoxycholate (Merck) up to 1.5% were used in splenic supernatants prepared at 7000 × *g* and 10 000 × *g* respectively.

Results

Ribonuclease activity and polysome content

Ribonuclease activity and polysome content were determined in the same 10 000 × *g* supernatant (S-10 fraction) of spleens obtained from untreated mice The ionic conditions were optimal for preservation of polyribosomes, a concentration of 10 mM $MgCl_2$ is inhibitory for pancreatic ribonuclease [14, 18] and splenic ribonuclease [19].

In normal spleens weighing between 100 and 130 mg the average proportion of ribosomes in polysomes was on a "low baseline" of 45% (± 3.5) with a corresponding ribonuclease activity of 14.6 (± 2.1) A_{260} units per mg protein, of which 51% (± 3.4) was inhibited. In the normal population of mice, a small group of about 10% showed considerable variation in the proportion of poly-

TABLE I

RIBONUCLEASE ACTIVITY AND POLYSOME CONTENT IN S 10 FRACTIONS OF SELECTED CONTROL MOUSE SPLEENS

Ribonuclease activity and the proportion of ribosomes in polysomes in the S 10 fraction were determined as described in Methods Values of ribonuclease activity obtained are the means of 4–6 separate determinations per spleen Standard deviation is given in brackets The percentage of polyribosomes was determined from samples which were run in duplicate The polysome profiles were reproducible with a range of 4% deviation The final concentration of $ClHgBzO^-$ was 1.8×10^{-3} M The eight samples were deliberately selected from the 10% minority to demonstrate the regulation of ribonuclease activity in samples with an increasing proportion of polyribosomes in untreated spleens

Spleen	Specific ribonuclease activity (A_{260} units per mg protein)		Percentage inhibited ribonuclease activity	Percentage ribosomes in polysomes
	Plus $ClHgBzO^-$	Without $ClHgBzO^-$		
1	11.9 (1.5)	5.4 (0.6)	55	42
2	14.7 (1.6)	6.8 (1.2)	54	49
3	14.0 (2.2)	6.7 (0.8)	52	50
4	14.3 (1.8)	7.4 (0.9)	48	52
5	14.7 (1.4)	6.6 (0.7)	56	53
6	17.4 (2.2)	4.3 (0.5)	75	65
7	17.9 (1.9)	2.3 (0.2)	87	69
8	14.4 (1.4)	2.3 (0.1)	84	69

somes as well as ribonuclease activity, whereas the spleen was also enlarged up to about 200 mg, due to some stimulation of the red pulp, as revealed by histological examination The data in Table I are not representative of normal mouse spleen, but have been deliberately selected from the 10% minority to make the point that in this group the percentage of polyribosomes correlates inversely with the actual endogenous ribonuclease activity The total specific ribonuclease activity as demonstrated by blocking the endogenous enzyme inhibitors through incubation in $ClHgBzO^-$ does not vary strikingly with the changes in polysomal content However, the actual ribonuclease activity (without

TABLE II

RIBONUCLEASE ACTIVITY AND POLYSOME CONTENT IN SPLENIC S 10 FRACTIONS OF PHENYLHYDRAZINE TREATED MICE

Conditions are the same as in Table I

Spleen	Specific ribonuclease activity (A_{260} units per mg protein)		Percentage inhibited ribonuclease activity	Percentage ribosomes in polysomes
	Plus $ClHgBzO^-$	Without $ClHgBzO^-$		
1	7.4 (0.5)	4.1 (0.5)	55	54
2	7.5 (0.4)	3.1 (0.3)	59	56
3	7.1 (0.4)	1.8 (0.3)	74	66
4	6.2 (0.4)	1.6 (0.2)	75	64
5	3.6 (0.5)	0.9 (0.2)	76	68
6	5.6 (0.6)	1.1 (0.3)	80	70
7	5.5 (0.4)	1.2 (0.2)	81	70

TABLE III

EFFECT OF $ClHgBzO^-$ ON THE YIELD OF POLYRIBOSOMES OF INFECTED SPLEENS

Sample experiment of one enlarged mouse spleen 14 days after infection with *P. berghei*, divided into equal parts. The S-10 fractions were prepared in buffer (50 mM Tris/HCl, 25 mM KCl, 10 mM $MgCl_2$, pH 7.6) with and without $ClHgBzO^-$ in one part, respectively. From a second spleen, purified polyribosomes were prepared (SP-6 fraction) and divided into equal parts, to one of which $ClHgBzO^-$ was added to a final standard concentration of $1.8 \cdot 10^{-3}$ M. Samples were incubated at 0 or 37°C for 30 min, and then analysed over sucrose gradients. The percentage of ribosomes in polysomes was measured.

Spleen fraction	Incubation medium	Percentage ribosomes in polysomes	
		0°C	37°C
S-10 fraction	without $ClHgBzO^-$	72	67
	plus $ClHgBzO^-$	57	35
SP-6 fraction	without $ClHgBzO^-$	56	45
	plus $ClHgBzO^-$	57	46

$ClHgBzO^-$, Table I) was frequently found to be lower in spleens with a higher polysomal content. Unless otherwise stated, all subsequent experiments with normal mouse spleen are from the 90% majority which have a relatively low baseline of polysomes. In spleens exhibiting rapidly proliferating erythropoietic tissue, both the total specific and the actual ribonuclease activity was less than half the normal values (Table II). The ribonuclease activity was inhibited up to 80% in S-10 fractions containing the relatively high proportion of 70% of polyribosomes. The enzymatic activity can be regulated by the amount of enzyme and inhibitor.

On the other hand, the total ribonuclease activity appears to be sufficient to cause a breakdown of polysomes as shown in Table III. Data of a sample experiment show that the proportion of polyribosomes from spleens of mice infected with *P. berghei* is relatively high when the isolation procedure is carried out in the absence of $ClHgBzO^-$ at 0 or 37°C (Table III). However, blocking of the endogenous ribonuclease inhibitor by including $ClHgBzO^-$ in the homogenisation buffer causes a drastic breakdown of large polyribosomes to smaller units and an overall loss of ribosomal material even at 0°C. $ClHgBzO^-$ did not affect purified polyribosomes (SP-6 fraction) which are obviously not contaminated with a ribonuclease inhibitor complex (Table III).

Effect of endogenous ribonuclease inhibitors

The inverse relationship between ribonuclease activity and polysomal content in splenic S-10 fractions led to the suspicion that additional inhibition of ribonuclease might result in higher polysomal yields. The 100 000 × *g* supernatant of rat liver is known to be rich in endogenous inhibitors [5,15,20,21]. To exclude any organ- or species specificity [11], the 100 000 × *g* supernatants of spleens of normal mice and of malaria-infected mice were used as the homogenisation medium in control experiments. The use of these supernatants as homogenisation medium did not affect the proportion of polysomes obtained from infected spleens, and even caused a reduction of the polysomal content in normal splenic S-10 fraction at 0°C (Table IV). However, incubation at 37°C of

TABLE IV

EFFECT OF DIFFERENT SOURCES OF RIBONUCLEASE INHIBITOR ON THE POLYSOME CONTENT IN NORMAL AND INFECTED SPLEENS

The 100 000 × *g* supernatants of rat liver, normal mouse spleen and malarial mouse spleen were used. Each spleen was divided into 2 or 3 equal parts. The S-10 fractions were prepared in homogenisation buffer, and the mentioned supernatants, incubated at 0 or 37 °C for 30 min, and analysed over sucrose gradients.

Spleen	Extraction medium	Percentage ribosomes in polysomes		
		0 °C	37 °C	Δ(0 °C–37 °C)
1 normal	buffer	45	61	+16
	liver supernatant	32	58	+26
2 normal	buffer	46	60	+14
	normal spleen supernatant	39	55	+16
	malaria spleen supernatant	31	55	+24
3 malarial	buffer	74	66	9
	liver supernatant	73	66	7
4 malarial	buffer	64	65	+1
	normal spleen supernatant	65	64	1
	malaria spleen supernatant	65	65	0

the S-10 fraction of normal spleens, which have a low proportion of polyribosomes (± 45%), caused a shift from monosomes to polyribosomes (Fig. 1) regardless of whether the S-10 fraction was prepared in homogenisation buffer or in liver supernatant and despite a relatively high actual ribonuclease activity

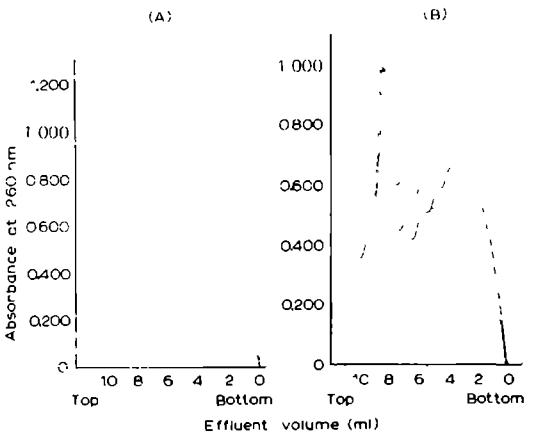

Fig. 1. Polysome profiles of mouse spleen after incubation of the S-10 fraction at 37 °C. The 10 000 × *g* supernatant was prepared as described in Methods. After incubation at 0 or 37 °C, 0.25-ml samples were analysed on 11.5 ml of an 8–10% linear sucrose gradient centrifuged for 90 min at 40 000 rev./min in the SW-40 rotor of a Spinco L2-65B centrifuge. Sedimentation from left to right. (A) Normal spleen. Incubation schedules: ——, 30 min at 0 °C; · · ·, 30 min at 37 °C; - - - - -, 60 min at 37 °C. (B) Malaria spleen. Incubation schedules: — – –, 30 min at 0 °C; - - - - -, 30 min at 37 °C.

TABLE V

EFFECT OF AURINTRICARBOXYLIC ACID ON RIBONUCLEASE ACTIVITY AND POLYSOME CONTENT

The S-10 fractions of normal spleens were incubated for 30 min at 0 or 37°C in presence of aurintricarboxylic acid and then analysed over sucrose gradients The free ribonuclease activity (without $ClHgBzO^-$) was determined as described in Methods (Standard deviation between brackets)

S 10 fraction from normal spleen	Percentage ribosomes in polysomes			Ribonuclease activity (A_{260} units per mg protein)
	0 C	37 C	Δ(0 C − 37 C)	
Buffer	47 (2 3)	67 (3 2)	+20	7 6 (0 6)
Aurin tricarboxylic acid 1 mM	47 (2 1)	46 (2 1)	−1	2 4 (0 3)
Aurin tricarboxylic acid 0 1 mM	47 (1 9)	46 (1 9(	−1	2 8 (0 2)

(Tables I and II) On the other hand, after prolonged incubation (1 h) at 37°C, both monosomal and polysomal material were reduced (Fig 1a).

Incubation at 37°C of the S-10 fraction having a high proportion of polyribosomes (+ 70%), obtained either from selected normal or from infected spleens did not significantly affect the polysomal yield and caused a shift of large polyribosomes to smaller polymers (Fig. 1b), although the actual ribonuclease activity was low We investigated whether in S-10 fractions of normal spleens, selected to a low baseline polysome content of ± 45%, the transformation to larger polysomes is related to an initiation process, using aurintricarboxylic acid as a blocking substance of the initiation step in protein synthesis [22]. It was found that this substance is able to prevent the shift from monosomes to polysomes (Table V) At the same time the free ribonuclease activity was inhibited

Effect of EDTA and detergents on the polysome content

The addition of EDTA only at a concentration of 0 1 mM to the homogenisation medium resulted in a somewhat higher polysomal yield at 0°C, although the ribonuclease activity was slightly stimulated Triton X-100 used in concentrations of 0.1 -1% did not yield any increase in polysomes and/or monosomes, and did not induce any changes in the polysomal profile when the spleen cells were disrupted in the presence of the detergent, nor when it was used in the 7000 or 10 000 × *g* supernatants Concentrations of up to 1% deoxycholate did not reveal changes in the polysomal profile, except for an occasional increase in the monosomal fraction.

Discussion

The results demonstrate that within a small percentage of the untreated control group, variations in splenic polysomal content and ribonuclease activity could be considerable, and paralleled the variations in splenic weight. The enzymatic activity seems to be regulated mainly by changes in the amount of inhibitor present in a resting spleen. In rapidly proliferating erythropoietic tissue, particularly from spleens of mice treated with phenylhydrazine, the enzymatic

activity is apparently regulated by changes in both the amount of inhibitor and of available enzyme. The activity of the endogenous ribonuclease inhibitor appears to increase with increasing proportion of polyribosomes. Comparing, however, the levels of total and free alkaline ribonuclease activity in controls and treated mice, it appears that the determination of the balance between ribonuclease and ribonuclease inhibitor under these ionic conditions is more instructive than determination of either of the separate components of the system. Despite a relative high free ribonuclease activity in control splenic extracts, a shift from monosomes to polyribosomes could be obtained by incubation at 37°C. This could mean that at high temperatures the initiation process continues at a higher rate than the elongation and translation process and increases the polysomal content in vitro. The absence of aminoacyltransferase I in control spleen [23] as a regulatory factor in controling protein synthesis at the translational level might favour the accumulation of polyribosomes The shift could be prevented by aurintricarboxylic acid, a substance blocking the initiation step in protein synthesis [22]. This substance also appeared to inhibit the ribonuclease activity considerably. The reversed shift, at 37°C, from heavier polyribosomes to lighter ones in an extract of anaemic spleen in the presence of very low ribonuclease activity will therefore be the result of an equilibrium shift in favour of the rate of translation. The polysomal integrity is preserves for hours when the translation process is restrained by low temperature. Melchers and Andersson [24] reported that redistribution of B-lymphocyte polysomes could only be induced with intact cells cultures in the presence of antigen for 1—3 h, but not with lysates. Whereas Nijhoff and Wieringa [25] routinely incubated reticulocytes for 5 min at 37°C to increase the polysomal content in lysates. Since the normal spleen is mainly erythropoietic, it is likely that the polysomal shift in splenic lysates is comparable to events in the reticulocyte system, and to the erythroid cells of the bone marrow [26]. Unlike uncommitted B-lymphocytes, sufficient stable globin messenger is present to safeguard the initiation process. It seems reasonable to conclude that the proportion of polyribosomes isolated from murine spleens is largely affected by at least two processes. (1) the balance between ribonuclease and its inhibitor and (2) the balance between initiation and translation processes in protein synthesis. The first process can be stabilized by ribonuclease inhibitor which is however, not in excess to the enzyme in the supernatants of rat liver [6] and murine spleens. Although the percentage of polysomes correlates inversely with endogenous ribonuclease activity, the amount of enzyme does not appear to play the expected decisive role and is not responsible for the low amount of polysomes extracted from control spleens, or from tissue cultures [27]. Possibly, the messenger and ribosomes, released after the translation process, lay open to the ribonuclease system. The role of ribonuclease in terms of integrity of the polysomes in the intact cells remains to be elucidated.

Acknowledgements

This study was supported by research grants from the World Health Organization. The able technical assistance of Mrs. Ingrid Mutsaerts is gratefully acknowledged.

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CHAPTER 3 PLASMODIUM BERGHEI: POLYRIBOSOME PROFILES IN THE SPLEENS OF INFECTED MICE

EXPERIMENTAL PARASITOLOGY 41, 83–88 (1977)

Plasmodium berghei: Polyribosome Profiles in the Spleens of Infected Mice

LAMBERT G. POELS

Department of Cyto-Histology, Faculty of Medicine, University of Nijmegen, Geert Grooteplein Noord 21, The Netherlands

(Accepted for publication 27 February 1976)

POELS, LAMBERT G. 1977. *Plasmodium berghei* Polyribosome profiles in the spleens of infected mice. *Experimental Parasitology* 41, 83–88. The distribution pattern of splenic polyribosomes in sucrose gradients was investigated in Swiss mice which had been inoculated with *Plasmodium berghei*-infected red blood cells. The onset of polyribosome formation in the spleen appeared to coincide with the first peak of parasitemia and to parallel the increasing pyroninophilia of the proliferating erythropoietic tissue. The polysome profile appeared to be similar after induction of anemia by phenylhydrazine and differs completely from the biphase profile which is obtained after antigenic stimulation with sheep red blood cells. The results suggest that the spleen is not involved in the synthesis of antibodies against a parasitic antigen during the course of a primary lethal infection with *Plasmodium berghei*.

INDEX DESCRIPTORS: *Plasmodium berghei*, Swiss mice; Spleen, Polyribosomes; Pyroninophilia, Erythropoiesis; Anemia; Phenylhydrazine; Sheep red blood cells; Sucrose gradients, Antigenic stimulation.

INTRODUCTION

The spleen of rodents is very heterogeneous in composition. The parasite, *Plasmodium berghei*, might act primarily as an antigen, stimulating the cells of the white pulp to produce antibodies, or stimulating the red pulp to greater erythropoietic activity through increased stimulation of erythropoietin production (Rencricca *et al.* 1974). The occurrence of immunosuppression in mice infected with *Plasmodium* is well documented (Salaman *et al.* 1969; Greenwood *et al.* 1971a; Sengers *et al.* 1971), although the degree of depression seems to vary considerably and may be transient in the case of self-limiting infection of *Plasmodium berghei yoelii* in BALB/c mice (Wedderburn 1974). *Plasmodium berghei berghei* is a fatal infection in Swiss mice, attended by massive proliferation of erythroid cells in the red pulp of the spleen (Kretschmar and Jerusalem 1963). As *Plasmodium berghei* might stimulate the proliferation of the white pulp as well as the red pulp, it was of interest to investigate the polyribosomal profiles after induction of a massive proliferation of the cells of the red pulp by phenylhydrazine treatment and after stimulation of the cells of the white pulp by administration of sheep red blood cells.

MATERIALS AND METHODS

Vertebrate host and parasite. Specific pathogen free (SPF) outbred Swiss and inbred BALB/c mice, approximately 8 to 10 weeks of age, were used in these studies. They were maintained on laboratory standard diet (Hope Farms Ltd.) and water *ad libitum. Plasmodium berghei berghei,*

ISSN 0014-4894

strain K173, has been maintained in this laboratory by serial weekly passage in Swiss and BALB/c mice. Routinely, the mice were inoculated intraperitoneally with 10^5 parasitized red blood cells. Parasitemias were determined in blood smears prepared from tail blood and stained with May-Grünwald-Giemsa. The numbers of parasitized red blood cells (p. RBC) and reticulocytes per 500 RBC were counted microscopically under oil immersion.

Phenylhydrazine treatment. Mice were injected subcutaneously with 0.2 ml of a neutralized 1% phenylhydrazine solution on several consecutive days. Spleens were removed 48 hr after the last injection. One part was used for histological observations, the other part for analysis of the polyribosome profile. The response of the animals was judged from reticulocytosis in blood smears.

Splenic polysomes. The preparation of splenic extracts and polysomes has been described in detail elsewhere (Jerusalem *et al.* 1971). Briefly, spleens were removed and placed immediately into ice-cold buffer, consisting of 50 m*M* Tris-HCl, 10 m*M* $MgCl_2$, and 25 m*M* KCl (TMK), pH 7.6. After homogenization the 10,000g supernatant was prepared (S-10 fraction).

TABLE I

Characteristics of Infection with *Plasmodium berghei* in Swiss Mice

Inoculum dose parasitized RBC	100	100,000
Pre-2% parasitemia (days)	6	3
First peak parasitemia on day	8–9	5–6
Percentage parasitized RBC at first peak parasitemia	22.5 ± 4.8	13.8 ± 5.7
Percentage of reticulocytes on day of first peak parasitemia	0.7 ± 0.8	0.9 ± 2.0
Onset development of polysomes on day	9–10	5–6

Aliquots of the S-10 fraction were analyzed on 11.5 ml of an 8–40% linear sucrose gradients centrifuged for 70 min at 40,000 rpm in an SW-40 rotor of a Spinco L2-65B centrifuge. The centrifuged gradients were analyzed by passing through the flow cell of a Zeiss recording spectrophotometer PMQ-II with the wavelength set at 260 nm. The proportion of polyribosomes in the total profile was determined planimetrically.

Immunization with sheep red blood cells. Sheep red blood cells (SRBC) were obtained from a stock suspension of whole sheep blood, diluted with an equal part of Alsever's solution. This stock suspension was kept in the cold and renewed every 4 to 6 weeks. Shortly before use, the cells were washed and resuspended in Alsever's. Unless otherwise stated, 1×10^8 SRBC were injected into a tail vein for immunization. Sera from immunized and control mice were heat inactivated (56 C, 30 min) and tested for anti-SRBC activity by hemagglutination or hemolysin assay in a microtiter system (Cooks, eng. U.S.A.). Hemolysis of 50% was recorded as the endpoint.

Experiments and Results

Splenic polyribosomal Profiles in the Course of Malaria Infection

In order to correlate the onset of any change in splenic polysomal profiles during the course of *Plasmodium berghei* infection, two groups of Swiss mice were infected intraperitoneally with 10^2 and 10^5 parasitized red blood cells, respectively. Parasitemia, reticulocytosis, splenic polysome profiles, and histological sections of six animals per day were investigated individually. The results, as summarized in Table I, show that a 1000-fold dilution of the inoculum prolongs the period needed to develop 2% parasitemia by 3 days and similarly delays the first peak of parasitemia. The onset of any change in the distribution pattern of polyribosomes is indicated by the appearance of di- and trimers and a general in-

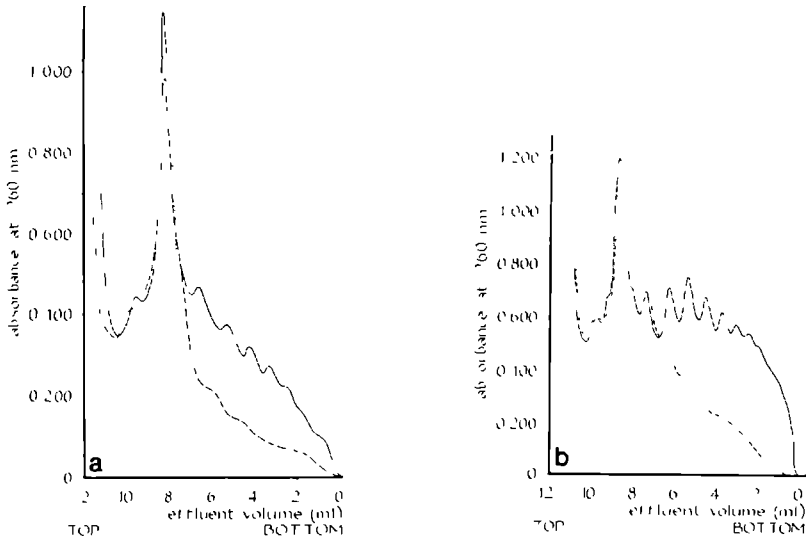

FIG. 1 (a), Polysome profiles of mouse spleen during the course of infection with *Plasmodium berghei*. The 100,00 *g* supernatants were analyzed over 8–40% linear sucrose gradients as described in Materials and Methods. Sedimentation from left to right. (---) Uninfected control spleen; (——) 1 day after the peak parasitemia; (-----) 3 days after the peak parasitemia. (b), Polysome profiles of mouse spleen after treatment with phenylhydrazine. Sedimentation from left to right. (---) Profile of a nonresponder with a small spleen; (——) profile of a responder with a large spleen.

crease in larger polyribosomes, as shown in Fig. 1a. It appeared that the profile was essentially identical to control values until peak parasitemia was reached. The gradually increasing amounts of heavier polyribosomes reached a steady level of 80% polyribosomes, defined as the proportion of polyribosomes in the total profile of monosomes plus polysomes, in samples obtained 14 days after malaria infection. The penta-hexa-, and heptasomes are the most abundant classes of the polysomes. Histological examination of sections of the spleen revealed that the increase in the yield of polyribosomes paralleled the proliferation of pyroninophilic erythropoietic cells in the red pulp of the spleen. These blood-forming stem cells in the spleen deliver reticulocytes to the peripheral blood 2 to 3 days later.

Phenylhydrazine Treatment

A rapidly developing severe anemia was induced by injections with phenylhydrazine on several consecutive days. The distribution pattern of polyribosomes obtained from activated enlarged spleens, 48 hr after the third injection (Fig. 1b) appears to be similar to that of malarial spleens. The sections were comparable: disappearance of lymphatic tissue in the white pulp, and a concomitant massive proliferative of erythropoietic tissue in the spleen. Some animals, however, did not respond adequately. Their spleen remained small and the proportion of polyribosomes was low (Fig. 1b). Such a group of nonresponders also occurs in malaria-infected mice. It is these mice which succumb early, i.e., between 7 and 14 days after infection.

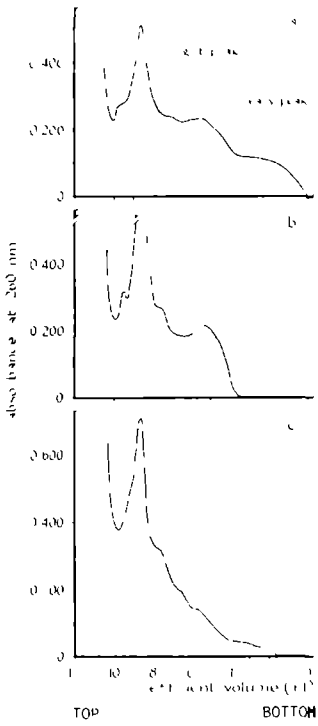

FIG. 2. Polysome profiles of mouse spleen after antigenic stimulation with sheep red blood cells (SRBC). Swiss mice were injected with 10^8 SRBC once a week. (a), Four days after single injection with SRBC. (b), Twelve days after single injection with SRBC. (c), Four day after the sixth weekly injection with SRBC.

Antigenic Stimulation with SRBC

A single injection of 10^8 SRBC causes a biphasic distribution of splenic polyribosomes after 3 to 4 days (Figs. 2a). The fifth and subsequent days were characterized by a continued decrease of the polysomes, the heavy clusters disappearing earlier than the light ones (Fig. 2b). The heavy cluster reappeared 3 days after a second injection with SRBC. Multiple, weekly injections with SRBC (Fig. 2c) did not stimulate the formation of polyribosomes further. In fact, the distribution pattern did not differ from that of control spleens. Repeated stimulation with SRBC did not result in an increase of serum antibody titer after the serum hemolysin had reached a titer of 1:4096. No plasma cells were noted in splenic sections of these animals, whereas plasma cells were abundant after one or two injections with SRBC only.

DISCUSSION

The development of different splenic polysomal profiles in response to either antigenic stimulation or induction of anemia by phenylhydrazine or malaria infection may be considered to reflect the physiological state of the cells, as was shown elsewhere (Poels 1976).

Analysis of the polysome profile during the course of a lethal *Plasmodium berghei* infection in Swiss mice revealed that two periods are distinguishable. During the first period, from the day of infection up to peak parasitemia, the spleen did not respond with formation of polyribosomes, independent of the inoculum.

However, during the second period, after peak parasitemia, progressive anemia stimulates the erythropoietic tissue in the spleen. The white pulp is replaced by hematopoietic cells with increasing pyroninophilia, due to an accumulation of ribosomal RNA. The polyribosomal distribution pattern appeared to be identical with that of spleens of phenylhydrazine-treated mice, which also exhibited a severely disturbed immunological response to SRBC (Poels, L. G., unpublished). The presence of different proportions of proerythroblasts, erythroblasts, normoblasts, and reticulocytes in the spleen might influence the concentration of the heavier polysomes, (Nijhof and Wierenga 1974). The distribution pattern, however, remains quite different from the polysome profile after antigenic stimulation with SRBC and reflects the synthesis of hemoglobin, rather than of antibodies against a parasitic antigen.

In contrast, antigenic stimulation with SRBC resulted in a biphasic distribution of

clusters of "light polysomes" and "heavy polysomes" which, according to Keuchler and Rich (1969), are involved in the synthesis and assembly of light and heavy polypeptide chains of an antibody molecule

This suggests that up to the time when peak parasitemia is reached the spleen is not involved in the synthesis of antibodies to the parasitic antigens This is in accordance with the immunologic quantitation by Sengers (1971) revealing that there is no increase in γ globulin during the course of a primary infection, and that the immunological responsiveness to heterologous erythrocytes and skin heterografts is increasingly impaired (Sengers *et al* 1971) At the height of parasitemia, at a time when non specific immunosuppression is known to occur (Salaman *et al* 1969, Greenwood *et al* 1971a, Sengers *et al* 1971, Greenwood *et al* 1971b), the lymphatic tissue in the spleen is greatly reduced (Kretschmar and Jerusalem 1963) A proliferation of plasma blasts in thymus dependent areas around the central arteriole, as noted by Moran *et al* (1973), was not observed On the contrary, a decreasing pyroninophilia was observed in the depleting white pulp of the spleen The reason for the difference might be that *Plasmodium berghei yoelii* causes a self limiting disease in BALB/c mice which lasts for about 3 weeks whereas the *Plasmodium berghei berghei* strain used in our studies always causes lethal infection The present results also agree with the findings of Dokow *et al* (1974) who reported a pronounced shift of the cell population in malarial rat spleens in the direction of hematopoiesis and away from immunocompetent cells Multiple reinfection with parasitized red blood cells, however, might result in a stimulation of cell mediated immunity, without a concomitant synthesis of antibodies in the spleen The polysomal profile of proliferating lymphocytes in the spleen would not be very different from that of an unstimulated, resting spleen, and therefore escape our attention

ACKNOWLEDGMENTS

I am grateful for the excellent assistance of Ingrid Mutsaerts and to Dr Margaret L Weiss for useful suggestions and valuable advice

This study was supported by a grant of the World Health Organization and by the International Collaborative Research Project on Malarial Immunopathology (ICRP/MII) supported by the Dutch Government

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CHAPTER 4 PLASMODIUM BERGHEI: IMMUNOSUPPRESSION AND HYPERIMMUNOGLOBULINEMIA

EXPERIMENTAL PARASITOLOGY 42, 235–247

Plasmodium berghei: Immunosuppression and Hyperimmunoglobulinemia

L. G. POELS AND CATHERINE C. VAN NIEKERK

Department of Cytology and Histology, Faculty of Medicine, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen, The Netherlands

(Accepted for publication 10 January 1977)

POELS, L. G., AND VAN NIEKERK, C. C 1977 *Plasmodium berghei* Immunosuppression and hyperimmunoglobulinemia *Experimental Parasitology* 42, 235–247 Fatal *Plasmodium berghei* infection in BALB/c mice caused permanent and complete depression of the mice's immune responsiveness to sheep erythrocytes beginning in the period of the first peak parasitemia and lasting until all the animals had died In the period of severe immunosuppression, tolerance to the sheep erythrocytes was induced, responsiveness to them was restored gradually when the infected mice were cured with chloroquine Considerably elevated levels of nonspecific antibodies, predominantly IgG, were produced during the period of immunosuppression The periarteriolar regions in the spleen, usually occupied by thymus-dependent lymphocytes, were progressively infiltrated by plasma cells during the period of immunosuppression, but also in malaria-immune mice which were boosted with parasitized red blood cells. There is a possible relationship between immunsuppression and hyperimmunoglobulinemia

INDEX DESCRIPTORS. Malaria, Protozoa, *Plasmodium berghei;* BALB/c mouse; Immunosuppression, Hyperimmunoglobulinemia, Anemia, Spleen, Plasma cell.

INTRODUCTION

Malarial infections are known to depress immune responses to diverse antigens. In mice, they reduce the antibody and splenic plaque forming cell response to sheep red blood cells (SRBC) (Salaman, Wedderburn, and Bruce-Chwatt 1969, Greenwood, Playfair, and Torrigiani 1971), prolong xenogeneic skin graft survival (Sengers, Jerusalem, and Doesburg 1971), enhance other infections (Cox 1975), and render animals more susceptible to lymphomagenic viruses (Wedderburn 1970). Reports on skin graft rejection and contact hypersensitivity as indicators of cell-mediated immunity are conflicting (Greenwood, Playfair, and Torrigiani 1971, Sengers, Jerusalem, and Doesburg 1971), and even when a single antigen such as SRBC was used, considerable variation in the degree of depression was observed, depending on the time when the antigen was injected relative to the stage of infection and on the host–parasite combination used (Wedderburn 1974). Different effects are exerted by *Plasmodium yoelii* and *P. berghei* in mice (Jayawardena, Targett, Leuchars, Carter, Doenhoff, and Davies 1975) or *P. berghei* in rats (Golenser, Spira, and Zuckerman 1975). It is debatable whether the immunosuppressive effects of the various parasites differed because of intrinsic differences or because of a difference in the length and severity of the resulting disease. If mice suffer from severe immunodepression, protective immunity could hardly be expected to develop, unless a strong response to the parasite might be the cause of the reduced responsiveness to other antigens. In mild infec-

ISSN 0014 4894

tions the synthesis of immunoglobulins directed against the malaria parasite was not found to be impaired (Greenwood 1974), while no increase in gammaglobulins was demonstrated in the case of *P berghei* infections in Swiss mice (Sadun, Williams, and Martin 1966, Sengers 1971) A discrepancy could exist between the amount of gammaglobulin demonstrable by biochemical methods and the functional level of immunoglobulin (Sengers 1971) The considerable variations in the degree of immunosuppression in different models, whether accompanied or not by changes in gammaglobulin levels, forced us to reinvestigate the mechanism of immunosuppression caused by the nonself limiting *Plasmodium berghei* infection in BALB/c mice The use of chloroquine enabled us to study also the recovery of the immune responsiveness

Materials and Methods

Mice

Experiments were carried out in BALB/c mice aged 10–20 weeks They were maintained on laboratory standard diet (Hope Farms Ltd) and water *ad libitum*

Malaria Infection

Mice were infected with *Plasmodium berghei* (strain K173) by intraperitoneal injection of 1×10 parasitized mouse red blood cells This malaria parasite has been maintained in our laboratory by serial weekly passage in BALB/c mice Parasitemia was determined in tail blood films stained with May–Grunwald/Giemsa The number of parasitized red blood cells and the number of reticulocytes per 500 RBC was counted microscopically The infection could be cured by adding 300 mg of chloroquine/liter to the drinking water

Phenylhydrazine Treatment

Mice were injected subcutaneously with 0 2 ml of a 1% neutralized phenylhydrazine solution on several consecutive days Animals which responded rapidly, as judged from reticulocytosis in blood smears, were used in the experiments 48 hr after the last injection, when the hematocrit value had dropped to about 20%

Immunization

Sheep blood provided by the central animal laboratory was stored at 4 C in Alsever's solution This stock suspension was renewed every 4 to 6 weeks Prior to use, cells were washed three or four times with Alsever's Unless otherwise stated, mice received 1×10^8 sheep erythrocytes intraperitoneally This antigenic dose appeared to yield the maximum number of plaque forming cells in the spleen Sera from immunized and control mice were heat inactivated (56 C, 30 min) and tested for anti SRBC activity by standard hemagglutination or hemolysin assays in a microtiter system (Cook Engineering, U S A) Titers are recorded as the mean and the standard error of the log$_2$-converted data, obtained from about 10 mice per day per sample Duplicate determinations were recorded

Plaque Forming Cells

The number of plaque forming cells (PFC) was determined by the method of Cunningham and Szenberg (1968), slightly modified according to Zaalberg, v d Meul, and v Twisk (1968)

Spleen cell suspensions were obtained on Days 3 to 7 after injection of sheep erythrocytes Samples of approximately 10 mice per day were used for triplicate determination of the number of PFC per 10^6 spleen cells The values are expressed as the mean of 10 individual triplicate determinations plus the standard error of the mean

Serum Immunoglobulins

Immunoglobulins concentrations were determined by quantitative rocket immu-

noelectrophoresis in 1% agarose in Gelman's high resolution buffer, pH 8.6, as described by Axelsen, Krøll, and Weeke (1973). Immunoglobulin standard and antiserum to murine IgM, IgG (7S, 1, 2A, and 2B), and IgA were obtained from Meloy Laboratories. The mean and standard error were calculated from 6 to 10 individual sera per sample per day, in duplicates. The indirect hemagglutination test for malaria was done as described by Meuwissen, Leeuwenberg, and Molenkamp (1972), with a lowest rejection threshold of 1:40 dilution.

Histology

Lymphoid tissue was fixed in Carnoy's solution. Sections of all tissues were routinely stained for cellular RNA content with methyl green pyronin. Blocks of tissue approximately 1 mm^3 were fixed in 0.1 *M* cacodylate-buffered 2.5% glutaraldehyde for 3 hr at 4 C and postfixed in 1% osmium tetroxide embedded in Epon 4:6. Ultrathin sections were double-contrasted with uranyl acetate and lead citrate and examined in a Philips E.M. 300.

Results

The Course of Malaria Infection

BALB/c mice infected with a standard inoculum of 10^5 parasitized red blood cells developed high parasitemias lethal to all mice within 30 days. The course of parasitemia and reticulocytosis are presented in Fig. 1A, showing that the parasitemia and the reticulocytosis rose to a high level shortly after the first peak parasitemia. In addition, during the first period from the day of infection up to 2 days after the first peak of parasitemia the parasites had invaded mainly oxiphilic erythrocytes, while in the period thereafter the parasites had penetrated mainly the reticulocytes. The malaria infection could be cured within 4 to 5 days by addition of 300 mg of chloroquine per liter to the drinking water (Fig. 1A), a procedure which led to a 100% immunity to rechallenge and reduced average spleen weights from 10 times to about twice the control values, while the average thymus weight returned from 15 mg ($\pm$ 11) to the normal value of 95 mg ($\pm$ 10) within 2 weeks after resolution of the parasitemia.

Responsiveness to Sheep Erythrocytes in Plasmodium berghei-Infected Mice, with and without Chloroquine Treatment

The design of the next experiments was to investigate how immune responsiveness to heterologous red blood cells (SRBC) is affected by *Plasmodium berghei* infection. In one group the infection was allowed to progress, and in the other group the parasitemia was eradicated with chloroquine treatment started at Day 14 of infection. Mice of both groups were injected with 10^8 SRBC at varying times in the course of infection and the number of plaque forming cells (PFC) in the spleen was determined on the Days 3, 4, 5, 6, and 7 after SRBC injection, in groups of 9–12 mice each per day (Fig. 2), while the anti-SRBC antibody titer was determined in Day 6 and 7 (Fig. 1B). When SRBC were injected 3 days before *P. berghei* inoculation (Fig. 2A; curve, day-3) a marked increase in the number of PFC in the spleen was obtained compared to the control values. In a second and third group, SRBC were administered at Days 7 and 14 after infection, respectively (Fig. 2A; curve, 7–14). The plaque response was completely eliminated at that time. The immunosuppression in noncured mice was not restricted locally to the spleen but apparently also occurred in other lymphoid organs as the anti-SRBC antibody titer in the serum was undetectable when the infection progressed (Fig. 1B), until the death of all mice. We did not observe even a delayed anti-SRBC response in chloroquine-treated mice, which received SRBC during the immunosuppressed period be-

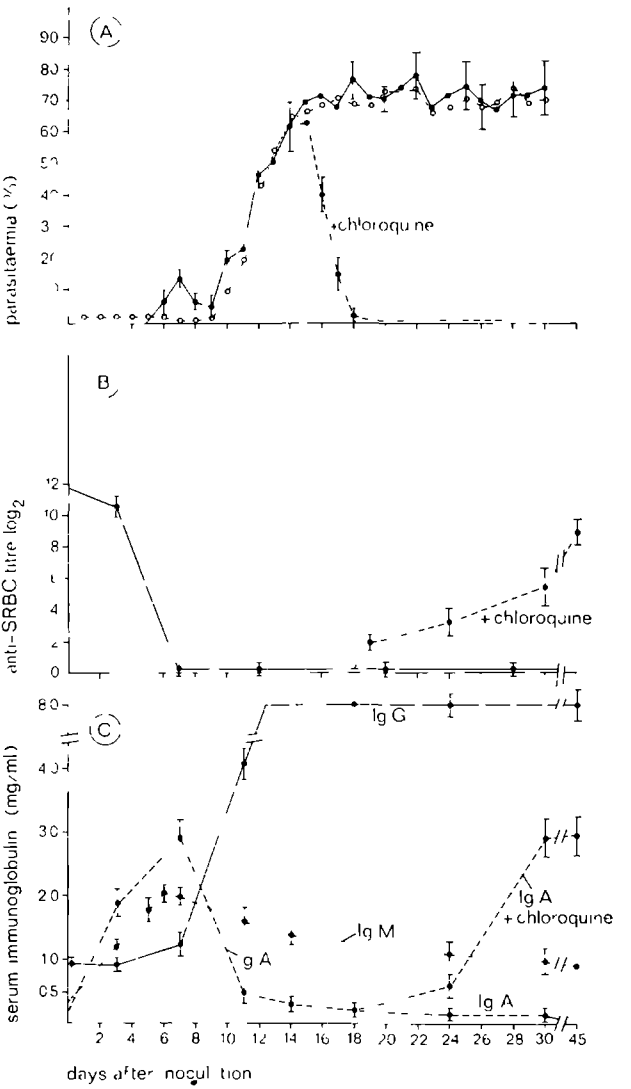

FIG 1 Determination of characteristics of *Plasmodium berghei* infection in BALB/c mice, with and without chloroquine therapy Vertical bars represent the standard error of the mean (A) Parasitemia and reticulocytosis in peripheral blood smears. Percentage of parasitized red blood cells without chloroquine therapy (—●—●—) and with chloroquine therapy started at Day 14 (----●----●----), percentage of reticulocytes without chloroquine treatment (----○----○----) (B) Immune responsiveness to SRBC. One group of *Plasmodium berghei*-infected BALB/c mice was kept on chloroquine drinking water from Day 14 postinfection (dashed line), while in the other group the infection was allowed to progress (solid line) SRBC (10^8) were injected at varying times during the course of infection and the mean logs of hemolysin titers were determined 6 to 7 days later in 9–12 mice. The titers are plotted in the curve in correspondence to the data of SRBC injection (C) Quantitative analysis of immunoglobulins in the serum of infected mice by means of rocket immunoelectrophoresis: As chloroquine treatment did not affect the IgM and IgG content in serum, only the data of IgM and IgG without chloroquine treatment were plotted.

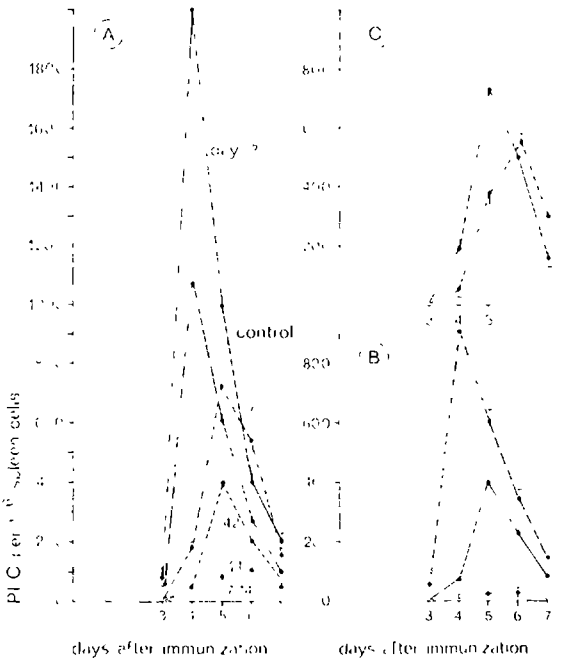

FIG. 2. The splenic immune response to sheep red blood cells (SRBC) in BALB/c mice infected with *Plasmodium berghei*, with and without chloroquine treatment. (A) In one group, 10^8 SRBC were injected 3 days before *Plasmodium berghei* inoculation (curve, day-3). In a second and third group 10^8 SRBC were injected on Day 7 and Day 14 after inoculation (curve, 7–14). In three other groups, 10^8 SRBC were injected at Days 21, 42, and 77 postinfection, respectively, (curve, 21, 42, and 77), but in these three groups the parasitemia was eradicated by chloroquine treatment from Days 14 to 21 postinfection. A noninfected control group without chloroquine treatment was used as a control. In all groups the number of plaque forming cells (PFC) per 10^6 spleen cells was determined on Days 3, 4, 5. 6, and 7 after SRBC injection. Vertical bars represent the standard error of the mean, determined in triplicates of 10 individual spleens per day sample. (B) Chloroquine was added to drinking water of a noninfected control group and of two groups of *Plasmodium berghei*-infected mice from Days 16 to 50 postinfection. One infected group was injected with 10^8 SRBC once at Day 16 and once at Day 42 of infection (- - - - - - - -); the second infected group (———) and the noninfected control group (— — —) received a single dose of 10^9 SRBC at Day 42 postinfection. The number of PFC was determined after the last SRBC injection, as described in (A). (C) A group of *Plasmodium berghei*-immune BALB/c mice was injected with 10^8 sheep erythrocytes (———), and a second immune group received simultaneously 10^8 SRBC plus 10^8 parasitized red blood cells (- - - - -). The number of PFC/10^6 spleen cells was determined as described in (A).

fore chloroquine treatment. In three other groups, in which the parasitemia was eradicated by chloroquine treatment from Days 14 to 21 after inoculation, SRBC were injected at Days 21, 42, and 77 after *P. berghei* infection, respectively (Fig. 2A; curve, 21, 42, and 77). The immunological responsiveness was slowly restored as judged from the PFC test with spleen cells (Fig. 2A) and from the anti-SRBC antibody titer in the serum (Fig. 1B).

In the next experiment it was investigated whether or not tolerance to SRBC could be induced in mice which suffered from a severe immunodepression. Chloroquine was added to the drinking water of

TABLE I

Effect of Phenylhydrazine Treatment on the Anti-Erythrocyte Response[a]

Days after injection of SRBC	Mean PFC per 10^6 spleen cells ± standard error		Mean hemolysin titer ($\log_2$ ± standard error)	
	Phenylhydrazine treatment	Control	Phenylhydrazine treatment	Control
3	13 ± 2	87 ± 19	0	3.9 ± 0.6
4	27 ± 11	789 ± 78	3.0 ± 0.3	6.3 ± 0.8
5	35 ± 14	568 ± 62	3.4 ± 0.4	7.9 ± 0.5
6	40 ± 18	164 ± 42	3.0 ± 0.6	10.0 ± 0.6
7	12 ± 3	82 ± 20	3.2 ± 0.6	11.2 ± 0.4
10	2 ± 2	43 ± 12	2.0 ± 0.3	8.7 ± 0.6

[a] Sheep red blood cells (5×10^7) were intraperitoneally injected in BALB/c mice which were made anemic with phenylhydrazine treatment as described in Methods

a noninfected control group and of two groups of *P berghei*-infected mice from Day 16 on after inoculation One infected group was injected with 10^8 SRBC in the period of immunosuppression (Day 16) and once again after recovery during chloroquine treatment (Day 42) The second infected group and the noninfected control group received a single dose of SRBC at Day 42 after inoculation Figure 2B shows that, after the last SRBC injection, no plaque forming cells in the spleen were produced in the group of mice that received SRBC twice, in contrast to the two other groups The hemolysin titers were in accordance with the results of the PFC test, being $\log_2$ 1.8 ± 0.3 for the group twice injected with SRBC and $\log_2$ 9.2 ± 0.5 for the group that received one injection of SRBC only in the recovery period This state of tolerance to SRBC could only be induced during the period of severe immunosuppression and anemia but not at the time of the first peak parasitemia. Simultaneous injection of 10^8 SRBC and 10^8 parasitized mouse red blood cells into mice immune to malaria only delayed the peak PFC by 1 day (Fig 2C) In order to check the specificity of the tolerance to SRBC, an unrelated antigen such as rabbit red blood cells was used Using the same experimental model as described above, 10^8 SRBC were administered at Day 16, while 10^8 RRBC were injected at Day 42 in the same group and in the control groups Seven days later the anti RRBC hemolysin titer in sera of 11 mice was determined and compared with a chloroquine-cured control group of nine mice which received only one injection of RRBC at Day 42 In both groups the anti RRBC titers were comparable being $\log_2$ 6.3 ± 0.5 and $\log_2$ 5.9 ± 0.5, respectively.

Immune Responsiveness after Phenylhydrazine Treatment

In mice in which severe anemia was induced by phenylhydrazine treatment (see Methods), massive proliferation of hematopoietic cells in the red pulp, concomitant with occasionally a nearly complete reduction of the white pulp, caused a fivefold increase of spleen weight within a week and paralleled a considerable thymic involution (from 95 ± 10 to 38 ± 8 mg) A reduced responsiveness to SRBC, as determined by the PFC assay in the spleen and the hemolysin titer in serum (Table I), occurred in the anemic period Attempts to induce tolerance to SRBC during phenylhydrazine anemia were unsuccessful. The immunosuppressed period usually lasted only some days as the mice recov-

ered very quickly from the effects of the drug. Phenylhydrazine was toxic to lymphoid cells *in vitro* as judged from a trypan blue dye exclusion test of incubated spleen cells. The immunoglobulin (IgM and IgG) concentration in serum was determined on Days 3, 7, and 14 after the last phenylhydrazine injection by means of rocket immunoelectrophoresis in groups of six mice each. Spleen sections of all these mice were examined for plasma cells. The serum immunoglobulin content remained unchanged and comparable to control values of untreated mice (Day 0 in Fig. 1C). No proliferation of plasma cells in the spleen was observed, in contrast to observations during malaria infection.

Serum Immunoglobulin Levels

The results of quantitative analyses by means of rocket immunoelectrophoresis of immunoglobulins in the serum of BALB/c mice infected with malaria are presented in Fig. 1C. During the period up to the first peak parasitemia there was a rapid increase of serum IgM and IgA levels. While during the second anemic period IgM levels gradually returned to an only somewhat elevated level of approximately 1 mg/ml independent of chloroquine treatment, the concentration of serum IgA dropped below normal levels and remained low until the animals were cured with chloroquine when a gradual return to approximately the same high levels as at the time of peak parasitemia was noted. Immunoglobulin IgG–7S levels increased considerably after peak parasitemia and remained elevated for a prolonged period, independent of chloroquine treatment.

In order to investigate whether or not the immunoglobulins formed in response to a primary malaria infection were antibodies against the parasites, 100 sera obtained during the course of infection were screened for anti-malarial activity by the indirect hemagglutination test (IHA) with a lowest threshold dilution of 1:40. Only three out of the 100 were positive, with a titer of 1:80 on Day 21, whereas the titers of boosted immune sera varied from 1:320 to 1:1280.

Spleen Histology

The histology of the spleen was investigated in 100 *Plasmodium berghei*-infected BALB/c mice of which 40 were cured with chloroquine treatment from Day 14 on. Sections were stained with methyl green pyronin. The gross modifications in the spleen, being a considerable reduction of the number and size of the white pulp follicles and a loss of the pyroninophilia of the germinal centers, concomitant with a hyperplasia of the red pulp, have been described in detail by Singer (1954) and by Kretschmar and Jerusalem (1963). However, in our BALB/c mice a proliferation and infiltration of pyroninophilic cells occurred in the perifollicular and in the thymus-dependent periarteriolar areas and remained extensive for a prolonged period (noticed up to 28 days postinfection). When the infection was cured with chloroquine, starting at Day 14 after infection, the reaction in the periarteriolar region gradually receded, while at the same time the white pulp follicles recovered and became considerably expanded. The recovered mice were immune to *Plasmodium berghei* (Poels, van Niekerk, Franken and van Elven, 1977). Repeated subsequent challenging of the malaria-immune mice with high doses of parasitized RBC (10^8 p RBC) caused a vigorous periarteriolar reaction (Fig. 3A). Electron microscopic investigations revealed that these pyronin-positive cells in the periarteriolar thymus-dependent areas were dividing plasmablasts and plasma cells (Fig. 3B).

Discussion

The first aspect of these experiments to be discussed concerns the immune responsiveness to sheep red blood cells (SRBC)

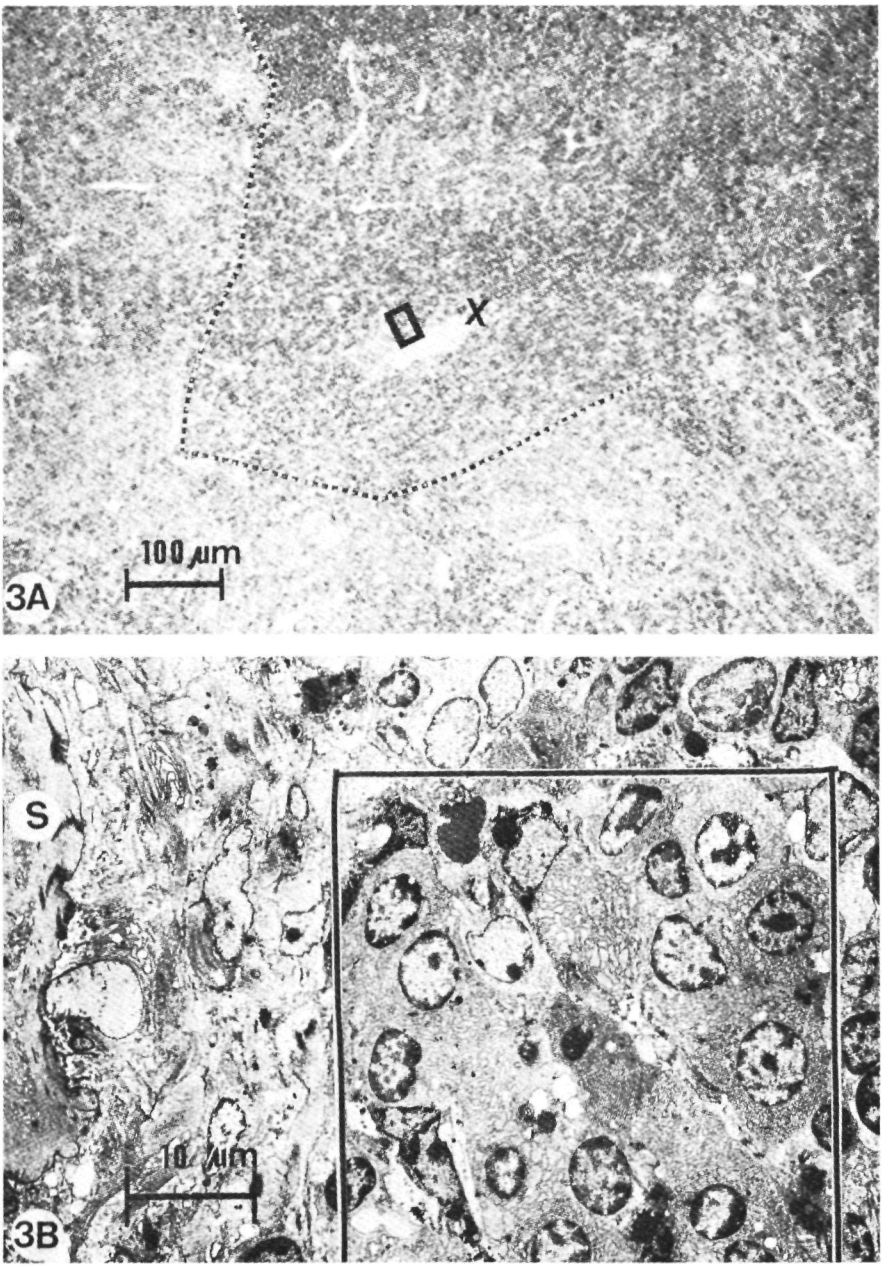

FIG. 3. (A) White pulp of the spleen of mice immunized with *Plasmodium berghei*, showing a confluent area of pyroninophilic cells immediately surrounding an arteriole (X) (Methyl green pyronin stained; interference filter of 510 nm). (B) Section showing the periarteriolar region: S = smooth muscles of the arteriole. Most of the cells within the inset are plasma cells.

in the course of the fatal *Plasmodium berghei* infection in BALB/c mice. The infection caused a permanent and complete suppression of the immune responsiveness to SRBC beginning in the period of the first peak parasitemia (Day 7) and lasting until all mice had died. The immunosuppression was not restricted to the spleen, as at the same time the anti-SRBC antibody titer in serum remained undetectable. Administration of SRBC during the period of immunosuppression followed by chemotherapeutical elimination of the parasites did not result in even a delayed formation of antibodies, while a booster with SRBC in the recovered mice did not recall SRBC memory cells but resulted in a tolerance to SRBC. Although the priming process was inhibited by the presence of parasitemia, confirming the data of Salaman, Wedderburn, and Bruce-Chwatt (1969) and of Greenwood, Playfair, and Torrigiani (1971), the proliferation of SRBC-primed cells was stimulated, as inoculation of mice with *P. berghei* 1 to 3 days after priming with SRBC resulted in an enhanced number of plaque-forming cells. Apparently, the parasites or their products can have an adjuvant-like effect. The tolerance to SRBC which was induced in the period of severe immunosuppression appeared to be specific as the response to an unrelated antigen (rabbit red blood cells, RRBC) was not inhibited in SRBC-tolerant mice. RRBC and SRBC evoke only a very slight antibody cross-reactivity (Playfair and Marshall-Clarke 1973). Our results are in contrast to the transient immunodepression caused by the self-limiting *P. yoelii* infection (Greenwood, Playfair, and Torrigiani 1971) and to the reported local state of suppression restricted to the spleen (Weidanz and Rank 1975). Tolerance was not induced in mice immunosuppressed by *P. yoelii* (Greenwood, Playfair, and Torrigiani 1971). Our results show that recovery of the immune responsiveness in *P. berghei*-infected mice could only be obtained after eradication of the parasitemia with chloroquine treatment which is possibly comparable to the spontaneous recovery of the PFC response in *P. yoelii* infected mice.

A second aspect to be discussed concerns a common feature for both strains, *P. berghei* and *P. yoelii*. It is the infiltration and proliferation of pyroninophilic cells, mainly of the plasma cell series, in the thymus dependent periarteriolar region occurring during the period of immunodepression. This reaction became extensive in our nonresolving *P. berghei* infection, although the white pulp follicles and germinal center activity were reduced considerably (Singer 1954, Kretschmar and Jerusalem 1963). The reaction receded gradually when the *P. berghei* infection was cured with chloroquine, as was also reported when the self-limiting *P. yoelii* infection regressed (Moran, de Rivera, and Turk 1973, Greenwood, Brown, de Jesus, and Holborow 1971). Repeated boosting of *P. berghei*-immune mice with high doses of parasitized red blood cells, however, evoked again a vigorous reaction of plasma cells in the thymus dependent periarteriolar lymphocyte sheath (PALS) as shown in the micrograph (Fig. 3B), concomitant with a considerable extension of the white pulp follicles and germinal centers (Kretschmar and Jerusalem 1963). The plasma cellular reaction in the PALS has been analyzed recently (Nieuwhenhuis and Ford 1976). Antigen-activated T cells and their progeny remain immobilized in the PALS for a long period. During this inductive period, B cells stream through and past the site of positively selected T cells in the PALS, thus allowing the required interaction between T and B cells in the presence of macrophages. Activated B cells which are going to differentiate to antibody-forming cells migrate toward the central PALS and finally via marginal zone-bridging channels into the red pulp (van Ewijk, Rozing, Brons, and Klepper,

1977) A continuous delivery of malaria antigens in the course of *P berghei* infection possibly caused a sustained streaming of B cells in the thymus dependent PALS leadings to a nearly complete occupation of that area as it occurred particularly in nude, athymic mice (van Ewijk personal communication) to which *P berghei* immunosuppressed mice are in a way comparable because of the severe thymic involution in that period

A third aspect to be discussed is the production of immunoglobulins in the course of infection Our results showed a remarkable rise of immunoglobulins (mainly IgG) during the period of immunosuppression in *P berghei* infection The immunoglobulins had low antiparasitic activity, as assessed by the indirect hemagglutination test (IHA) and by fluorescent studies (unpublished observations) This is in accordance with the reported low antimalarial antibody titers in *P berghei*-infected mice and differs from the elevated levels of antiparasitic antibodies in *P yoelii* (Javawardena, Targett, Leuchars, Carter, Doenhoff, and Davies 1975) Although the antimalarial antibody titer was low a certain amount of these immunoglobulins has specificity to malarial antigens, as immune complexes were detected in the glomeruli of kidneys of 14 day infected mice (Poels, van Niekerk, Pennings, Agterberg, and van Elven, 1977), confirming results of Boonpucknavig, Boonpucknavig and Bhamarapravati (1973) The deposition of immune complexes in the glomeruli possibly causes a depletion of malaria specific antibodies in the serum This, however, does not elucidate why the bulk of immunoglobulins produced during the period of immunosuppression did not show avidity for the parasites in contrast to antibodies obtained from hyperimmune mice The mechanism responsible for the formation of nonspecific immunoglobulins in malaria infection is unknown, but is possibly related to the phenomenon of immunodepression Several factors may play a role in causing the state of immunodepression (1) As erythropoietic and antibody forming cells probably are derived from a common precursor cell (Doria 1969), severe anemia might affect the immune responsiveness However, the unresponsive state caused by phenylhydrazine treatment differed from the malarial model in that the depression was very temporary, a tolerance to SRBC was not induceable, and no increase in immunoglobulins was noticed Therefore the anemia, even accompanied by thymic involution due to phenylhydrazine, is not sufficient to account for the severe disturbance of the immune responsiveness in *P berghei* infection (2) Blocking or preempting the reaction of any one of the cell types (T cells, B cells and macrophages) cooperating in the immune response will result in inhibition of the production of antibodies to SRBC The T cell activity in *P berghei* infection was severely reduced as judged from thymic involution (see also Jerusalem 1965), a decreased number of SRBC-reactive T cells in the peripheral circulation and in lymphatic organs (Kiettli and Nussenzweig 1974), and a depressed proportion of PHA-sensitive cells (Javawardena, Targett, Leuchars, Carter, Doenhoff, and Davies 1975, Golenser, Spira, and Zuckerman 1975)

The differentiation and maturation of IgA producing cells is controlled to some extent by the thymus (Butler and Oskvig 1974) The drop in IgA level during the immunosuppressed period and its rise after chloroquine treatment, when the thymus was also repopulated and the immune responsiveness to SRBC restored supports indirectly the idea that the T cell function in *P berghei* infected mice is reduced On the other hand, mopping up of serum IgA by malarial antigens and subsequent deposition of these immune complexes in the kidney glomeruli which appeared to be positive for IgA (Poels, van Niekerk, Pennings, Agterberg and van Elven, 1977)

may be partly responsible for the IgA drop during the immunosuppressed period. The high production of mainly IgG immunoglobulins in the period of immunosuppression might be the consequence of reduced T cell function deregulating the normal control mechanisms for antibody producetion. A deletion of T suppressor cells as well as T helper cells would affect consequently the production of antibodies and their specificity.

Alternatively, preemption of B cell function by malarial antigens and/or a malarial B cell mitogen, as recently demonstrated in *P. falciparum*-infected children (Greenwood and Vick 1975), would also cause elevated (nonspecific) antibody production with a concomitant depression of the immune response to newly introduced antigens (e.g., SRBC). It would also explain the adjuvant-like stimulation of proliferating SRBC-primed cells occurring when mice were first primed with SRBC and 3 days later infected with *P. berghei*, and even the induction of unresponsiveness and tolerance could be explained by the existence of such a B cell mitogen, like poke weed mitogens and lipopolysaccharides, as inducers of terminal differentiation and immunoglobulin synthesis (Andersson, Sjoberg, and Moller 1972a, b), when, for example, lymphocytes are exposed first to a mitogen and then to an antigen. Although similar phenomena can be explained by T cell mitogens (*in vitro*) (Dutton 1975) the existence of them is not likely, as *Plasmodium berghei* infection elicited only a very limited T cell activation (Jayawardena, Targett, Leuchars, Carter, Doenhoff, and Davies 1975).

The data yet available do not permit any definitive conclusions about which of the factors discussed above are responsible for all the phenomena as immunodepression, hyperimmunoglobulinemia, and tolerance induction because absence or unresponsiveness of any one population of specific cells (either T or B) can lead to an operational immunological unresponsiveness and tolerance at the whole animal level. The disturbance of the immune response may be the result of a combination of at least three factors: (a) severe depletion of T cells, (b) nonspecific recruitment of B cells by a mitogen factor, and (c) consequent disruption of interaction with macrophages.

Acknowledgments

We wish to thank Professor Dr. C. Jerusalem for his help throughout this study, Dr. Margaret L. Weiss for useful suggestions and valuable advice during preparation of the manuscript, and Dr. P. Jap and co-workers for their help with the histological investigations. We are much obliged to Dr. J. Meuwissen and Mrs. A Leeuwenberg for carrying out the indirect hemagglutination test. We acknowledge the assistance of Mrs. V. van der Sterren and of Mr. J. Reitsma and collaborators (Central Animal Laboratory).

This study was supported by a grant from the World Health Organization and by the International Collaboration Research Project on Malarial Immunity and Immunopathology (ICRP/MII) supported by the Netherlands, Government.

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CHAPTER 5 PLASMODIUM BERGHEI: SELECTIVE RELEASE OF "PROTECTIVE" ANTIGENS

EXPERIMENTAL PARASITOLOGY 42, 182–193 (1977)

Plasmodium berghei: Selective Release of "Protective" Antigens

L G POELS, CATHERINE C VAN NIEKERK, AND MAGDA A M FRANKEN

Department of Cytology and Histology, Faculty of Medicine, Catholic University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen, The Netherlands

AND

E H VAN ELVEN

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology University of Amsterdam, Amsterdam, The Netherlands

(Accepted for publication 13 December 1976)

POELS, L G, VAN NIEKERK, C C, FRANKEN, M A M, AND VAN ELVEN, E H 1977 *Plasmodium berghei* Selective release of protective antigens *Experimental Parasitology* 42, 182–193 BALB/c mice infected with *Plasmodium berghei* produced high levels of immunoglobulins after the first peak parasitemia at which time the malaria parasites were mainly present in reticulocytes and not in oxyphilic red blood cells Attempts at active immunization with intact parasitized reticulocytes were successful, while immunization with parasitized oxyphilic erythrocytes failed completely The antibodies produced in response to immunization reacted with free parasites and penetrated only those reticulocytes which were infected with three to four larger parasites Although the first peak mortality in acute infection was prevented with immune serum, the infection could not be aborted by immune serum or spleen cells Nevertheless in 60% of syngeneic mice protection was transferred by giving them multiple injections of immune spleen cells during a period of 4 weeks prior to infection

INDEX DESCRIPTORS Malaria, Protozoa, *Plasmodium berghei — P berghei berghei*, BALB/c mice, Protective immunization, Oxiphilic, polychromatophilic erythrocytes, Immunofluorescence, Immunoperoxidase, Transfer of serum, Spleen cell transfer

INTRODUCTION

Plasmodium berghei infection in mice causes a high degree of parasitemia and proves fatal within 30 days, although the survival pattern appears to be polyphasic and somewhat dependent on the inoculum dose (Sengers, Liem, and Doesburg 1971) A severe degree of depression of the immunological responsiveness to heterologous erythrocytes and skin grafts was observed during the period after the first peak parasitemia (Sengers, Jerusalem, and Doesburg 1971) The depression of the immunological responsiveness together with an enhanced production of heterospecific antibodies, possibly as a result of the severe T cell depletion (Weiss, M L, in preparation) and/or nonspecific recruitment of B cells by a malarial mitogen (Greenwood 1974, Greenwood and Vick 1975), might have important consequences for the response to malarial antigens released during that period The building up of protective immunity to the parasite would be impaired only if the malarial antigens released before the period of im-

ISSN 0014 4894

immunosuppression were "nonprotective" antigens or if, alternatively, 'protective antigens" were not available to the host in sufficient quantities In the present study we have attempted to determine which stages during the course of infection were able to elicit protective immunity The character of the immunity was further explored with serum and cell transfer studies, immunofluorescence, and immunoperoxidase For comparison the response to a single and to multiple administration of xenogeneic red blood cells was studied

MATERIALS AND METHODS

Vertebrate Host and Parasite

Specific pathogen free inbred BALB/c mice between 10 and 20 weeks of age were used in these studies They were maintained on laboratory standard diet (Hope Farms Ltd) and water *ad libitum Plasmodium (berghei) berghei* (strain K 173) has been maintained in this laboratory by serial weekly passage in BALB/c mice Routinely, the mice were inoculated intraperitoneally with 10^5 parasitized red blood cells Parasitemias were determined in blood smears prepared from tail blood and stained with May-Grunwald/Giemsa The number of parasitized red blood cells (p RBC) and reticulocytes per 500 RBC was counted microscopically An infection could be cured by adding 300 mg of chloroquine/liter to the drinking water

Preparation of Xenogeneic Red Blood Cells Hemagglutination and Hemolysin Tests

Blood from a New Zealand rabbit was provided by the central animal laboratory and stored at 4 C in Alsever's solution These stock suspensions were renewed every 4 to 6 weeks Prior to use, cells were washed three times with Alsever's Unless otherwise stated, mice received 5×10^8 rabbit red blood cells (RRBC) This amount of RBC yielded the maximum number of plaque forming cells in the spleen 4 days later Sera of immunized and control mice were heat inactivated (56 C/ 30 min), and tested for anti-red blood cell activity by standard hemagglutination and hemolysin assays in a microtiter system (Cook Engineering, U S A) Titers are recorded as the mean and the standard error of the log$_2$ converted data, obtained from 8 to 11 mice per day per sample Fresh frozen guinea pig sera (−20 C) were used as a source of complement and, when necessary, absorbed with red blood cells from mice and rabbit

Fractionation of IgG and IgM

The 19 S and 7 S antibodies were isolated from sera by Bio gel A 0 5 m (Bio-Rad Laboratories, Richmond, California) gel filtration through a K50/60 column (Pharmacia, Uppsala, Sweden) as described by Jansen, Koene, van Kamp, Tamboer, and Wijdeveld (1975) Reconcentrated fractions were tested in a two dimensional immunodiffusion system against specific antisera to IgM and 7 S IgG (Meloy Laboratories, Springfield, Virginia) according to the method of Ouchterlony, or by rocket immunoelectrophoresis (Axelsen, Krøll, and Weeke 1973) IgM was present only in the first peak (Fig 1), IgG was predominantly present in the third peak Sera and column fractions were reduced with 2 mercaptoethanol (2-ME), or with dithiothreithol (DDT) as described by Domel, Ropars, and Salmon (1973), in order to distinguish between IgM and IgG antibodies

Spleen Cell Transfer

Spleens from immunized mice were quickly removed and immediately gently homogenized in a loosely fitting Teflon–glass homogenizer with a "screwed" pestle, in presence of 10 ml of TC 199 medium (Difco) supplemented with 17 m*M* Tris–HCl and 5% fetal calf serum (Flow),

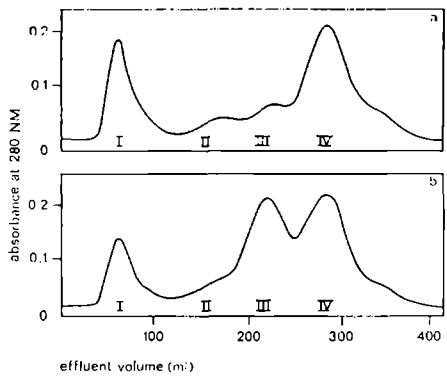

FIG. 1. The 19 S (peak I) and 7 S (mainly peak III) antibodies isolated from sera by Biogel A-0.5 m gel filtration. (a) Sera from mice immunized with 6 weekly injections of RRBC; (b) sera from mice 12 days after infection with *Plasmodium berghei*. The log of I_0/I absorbance was monitored at 280 nm.

pH 7.4 Cell clumps were permitted to settle for 2 min, the supernatant was sieved and centrifuged at 1000 rpm for 8 min, and the cells were suspended in medium without calf serum. Viable nucleated cells were counted. The spleen cells were transferred intravenously in a volume of 0.2 ml.

Immunofluorescence

Rabbit anti-mouse immunoglobulins were isolated and conjugated with fluorescein isothiocyanate (FITC) as described previously (Jerusalem, Weiss, and Poels 1971). Washed parasitized oxiphilic or polychromatophil red blood cells were incubated in hyperimmune serum at 0 C for 30 min, washed three times with TC-199 medium, and incubated in FITC-conjugated rabbit anti-mouse Ig at 0 C. Cell suspensions were examined under the fluorescence microscope.

Immunoperoxidase

Infected red blood cells were washed and fixed for 10 min in cacodylate buffer–1.7% glutaraldehyde (320 mOsmoles). The washed, fixed cells were incubated in malaria-immune serum (30 min 0 C), washed in PBS, reacted with rabbit anti-mouse IgG (absorbed with parasitized mouse RBC), washed in PBS, and incubated in peroxidase-conjugated pig anti-rabbit IgG (Meloy) for 30 min, 0 C. Subsequently, the cells were processed for peroxidase staining using diaminobenzidine and H_2O_2 (Graham and Karnowsky 1966), postfixed in 1% osmium tetroxide according to Millonig (1961), dehydrated, embedded in Epon, and processed for electron microscopy. Sections were stained with uranyl acetate and lead citrate.

EXPERIMENTS AND RESULTS

Active Immunization

Although BALB/c mice infected with a standard inoculum of 10^5 parasitized red blood cells (p. RBC) developed high parasitemias lethal to all mice within 30 days, a considerable amount of mainly IgG globulins (peak III in Fig 1b) was produced after the first peak parasitemia. Most of these globulins had no detectable avidity for the parasite (Poels and Niekerk, 1977. However, complete immunity to the parasite could be established by chloroquine therapy applied at the right time, as outlined in the following experiments:

(*a*) *Timing of chloroquine therapy.* Two groups of 100 mice each were infected with 10^5 parasitized erythrocytes on Day 0. In one group the parasitemia was aborted by addition of chloroquine to the drinking water (300 mg/liter) on Day 7 for 1 week; the second group was cured by addition of chloroquine on Day 14, also for one week. Both groups of mice cleared the infection and received normal drinking water thereafter. Both groups were challenged on Day 42 with 10^5 parasitized RBC. All mice in the first group (Day 7) developed normal infections and died within 30 days, whereas in the second

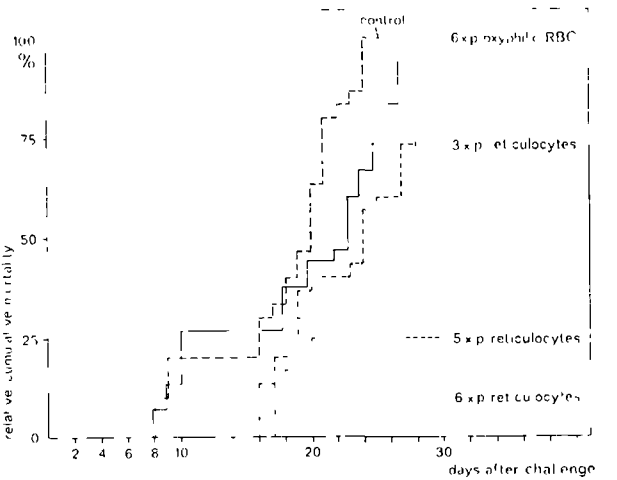

FIG. 2 Immunization of chloroquine-protected mice (40 per group) with multiple injection of 5×10^7 parasitized oxiphilic and polychromatophilic red blood cells. All groups were challenged with 10^7 RBC parasitized with *Plasmodium berghei* 2 weeks after return to normal drinking water.

group (Day 14) only one of the original survivors died. The surviving mice did not develop a detectable parasitemia, not even after a second challenge with 10^7 p.RBC. This result may indicate that the first 7 days of infection, when only mature oxiphilic red blood cells were infected, did not yield a sufficient quantity of parasitic antigen or that the antigens released were qualitatively insufficient, in contrast to those released during the second week when the degree of both parasitemia and reticulocytosis was approximately 50% and the parasites were almost exclusively present in reticulocytes.

(*b*) *Pretreatment with specific stages.* Five groups of 40 mice each were given 300 mg of chloroquine/liter in drinking water during a period of 6 weeks during which time they were once or repeatedly injected with 5×10^7 p.RBC obtained either from the oxiphilic stage (Day 7) or from the reticulocytic stage (Day 14) of the infection. Under these conditions parasitic growth or metabolism is not permitted so that the quantity of antigen is controlled. The mice were challenged with 10^7 p RBC 2 weeks after return to normal drinking water. The cumulative mortality is represented in Fig. 2. The results show that a 25% survival rate was obtained after three immunizing injections, and a 95% survival rate after 6 weekly injections with parasitized *reticulocytes.* The survivors never developed a parasitemia even upon a second challenge with 5×10^7 p.RBC given 1 month later. In contrast, all animals of the group which received 6 weekly injections of parasitized oxyphilic red blood cells succumbed within 30 days. Congruent with these results were the histological observations that the white pulp in the spleens of mice that had received six injections with parasitized reticulocytes showed a vigorous and sustained germinal center response, while only very weak stimulation was caused by multiple injections with parasitized oxiphilic red blood cells. Attempts at immunization were completely unsuccessful when the injected parasitized reticulocytes were previously hemolysed with antibod-

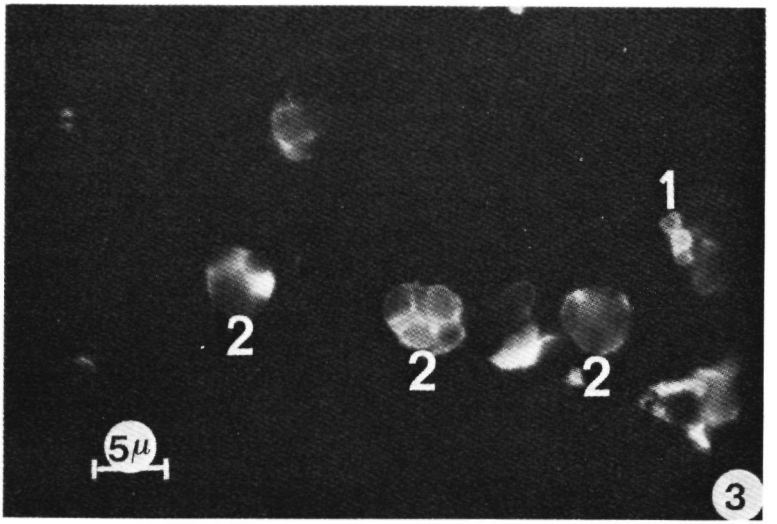

FIG. 3. Membrane immunofluorescence of unfixed reticulocytes parasitized by *Plasmodium berghei* obtained on Day 14 of infection. The washed p.RBC were incubated in hyperimmune serum at 0 C and thereafter in FITC-labeled rabbit anti-mouse immunoglobulins. Note that only free parasites (1) and only those reticulocytes (2) which contain three to four larger parasites simultaneously are strongly positive.

ies and complement and washed thereafter, a procedure which left the still-viable parasites within the red cell membrane (Jerusalem and Eling 1969) but probably allowed the escape of "soluble" malarial antigens.

Immunofluorescence and Peroxidase

The antibodies produced in response to the immunization procedure with parasitized reticulocytes were investigated with indirect immunofluorescence. A total absence of fluorescence of parasites within mature oxiphilic red blood cells (Day 7) was noticed. The free parasites and those reticulocytes which contained three to four larger parasites were strongly positive as shown in Fig. 3. At an ultrastructural level it appeared that wherever the reticulocyte membrane had become "damaged" and the parasites had become accessible, deposition of immunoperoxidase-positive material occurred within the reticulocyte at the point where the parasite is closest to the reticulocyte membrane (Fig. 4). Parasites within an intact reticulocyte were not coated with peroxidase conjugated antibodies, whereas that region nearest to the parasite just outside the reticulocyte was heavily coated with labeled antibodies (Fig. 5).

Transfer of Malaria Immunized Spleen Cells and/or Serum

As a control experiment for the spleen cell transfers rabbit red blood cells (RRBC) were used as a nonpathogenic, nonproliferating antigen to prime spleen cells. As shown in Table I the transferred spleen cells, obtained from RRBC-boosted mice (no. 2 in Table I), could not produce detectable levels of antibodies in the recipients, nor could the transferred anti-RRBC memory be recalled by a simultaneous or delayed administration of RRBC to the recipients. Within a dose range of

10^3 to 5×10^7 transferred spleen cells, the negative results were independent of the route of injection, of timing of collection of the donor cells after priming with RRBC, or of the challenge dose of RRBC in order to recall the memory cells. However, antibodies were detectable in the recipient only 24 hr after intravenous injection of 2×10^8 spleen cells (approximately one spleen equivalent) primed with RRBC 4 days previously (no. 3b in Table I). This titer of antibodies was reduced rapidly to undetectable levels within 3 days. Apparently the transferred plasma cells just secreted their antibodies without further proliferation.

Mice immunized to *Plasmodium berghei* as described above were used as donors of immune serum and spleen cells. The malaria immune serum was positive in the indirect immunofluorescence test, as shown in Fig. 3. The design of the experiment was to investigate whether or not the immune serum and/or spleen cells were able to affect an acute *P. berghei* infection. Therefore, groups of 40 BALB/c mice each were infected with *P. berghei* on Day 0. Five days later, when the parasitemia had reached a degree of about 2%, one group was injected with 10^8 hyperimmune spleen cells and a second group with 0.3 ml of hyperimmune serum from the same donor group. Control groups received serum and spleen cells from RRBC-immunized donors. Parasitemia in the recipients were monitored by daily blood smears, and the mortality was registered twice a day. As shown in Fig. 6, neither immune serum nor immune spleen cells affected the degree or the course of parasitemia, nor was the cumulative mortality affected by transferred immune spleen cells. While immune serum consistently prevented the first peak mortality, the mice succumbed somewhat faster during the second phase of the infection. When spleen cells plus immune serum were injected the same effect as with immune serum alone was obtained. The results were not improved when multiple injections of cells or serum were administered for a longer period, and when a single shot of serum and or spleen cells was given on the day of challenge, no difference was noticed between spleen

TABLE I

Transfer of Spleen Cells Primed with Rabbit Red Blood Cells (RRBC)[a]

Intravenous injection of 5×10^8 RRBC and/or RRBC-committed spleen cells	Mean $\log_2$ of antibody titer plus standard error	
	Hemolysin	Hemagglutinin
(1) Primary response to RRBC	6.2 ± 0.6	4.8 ± 0.5
(2) Secondary response to RRBC	6.1 ± 0.2	11.2 ± 0.3
(3a) Transfer of RRBC-committed spleen cells (10^3–5×10^7)	0	0
(3b) Transfer of 2×10^8 RRBC-committed spleen cells primed 4 days previously	2.3 ± 0.2 (0)	3.6 ± 0.3 (0)
(4) Transfer of 5×10^7 RRBC-committed spleen cells plus RRBC simultaneously or 10 days later	5.2 ± 0.4	4.8 ± 0.2

[a] Adult BALB/c mice were injected intravenously with 5×10^8 rabbit red blood cells either once or six times. The hemolysin and hemagglutinin titers were determined 7 days after the last injection. The mean titer and its standard error were obtained from 9 to 12 individual bleedings. RRBC-committed spleen cells were obtained from RRBC-boosted mice as described in Methods and injected intravenously in syngeneic BALB/c mice. The antibody titer was determined 7 days later. In no. 3b the titer was determined after 1 day and 4 days (in parentheses).

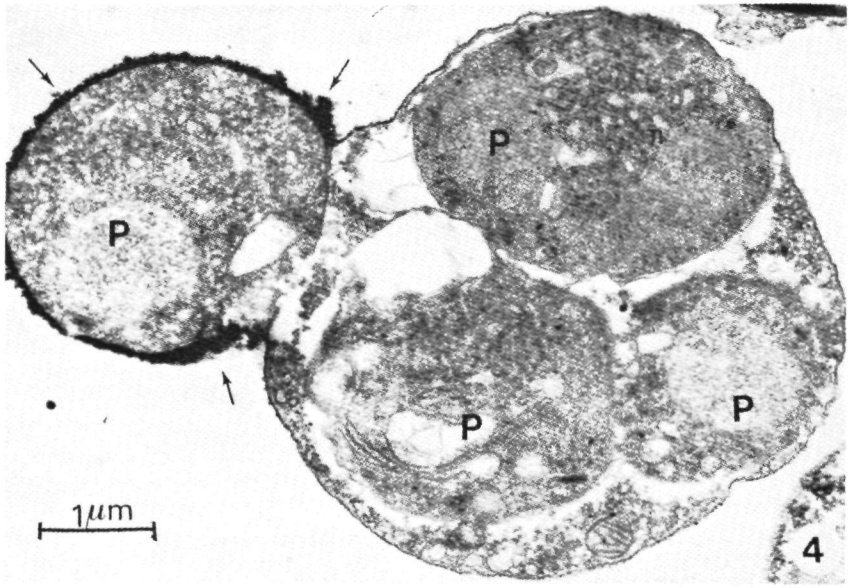

FIG. 4. Intact, *Plasmodium berghei*-infected reticulocyte, 14th day of infection, incubated in hyperimmune anti-malarial serum, rabbit anti-mouse IgG, and peroxidase-conjugated pig anti-rabbit IgG. Arrows indicate deposition of peroxidase-conjugated antibodies on that part of the parasite extruded from the intact reticulocyte.

cells obtained on Days 7, 21, or 42 after boosting of the hyperimmune donor mice.

Priming with Immune Spleen Cells

Although an acute infection could not be cured by transferred serum and/or spleen cells, the results were improved when noninfected recipients were "pretreated" with immune spleen cells. Transferred isologous committed spleen cells might multiply to an effective level in the recipient, or, alternatively trigger and commit host cells to the same specificity. Forty normal BALB/c mice were injected intravenously four times with 2×10^7 hyperimmune spleen cells (prepared 21 days after boostering) at weekly intervals under chloroquine conditions. Even without chloroquine, the spleen cell suspensions were not able to induce parasitemia in normal mice. Two weeks after the fourth injection they were challenged with 10^5 p.RBC. A control group of 40 mice was pretreated with RRBC-immunized spleen cells under chloroquine conditions. Of the 40 mice pretreated with malaria immune spleen cells, 24 (60%) survived the first challenge infection and did not develop a detectable parasitemia. The other 40% died with normal heavy parasitemias. A second challenge with 10^7 p.RBC, 1 month later was survived by 23 out of 24 mice. All control mice died within 30 days.

DISCUSSION

The first aspect of these experiments to be discussed concerns the excessive production of apparently heterospecific immunoglobulin (mainly IgG) during the period of severe immunosuppression, i.e., during and after the first peak parasitemia of *P. berghei* infection in BALB/c mice (Poels and Niekerk, 1977). The low antiparasitic antibody titer noticed by

Jayawardena, Target, Leuchars, Carter, Doenhof, and Davies (1975) in *P. berghei-infected* CBA mice is in agreement with the low specificity of the antibodies produced in acute infections in our BALB/c mice and differs therefore markedly from nonfatal plasmodial infections in mice and rats. A deregulation of the normal control mechanisms for antibody production might be caused by a deletion of suppressor T cells as a consequence of thymic involution during the period of immunosuppression (Weiss, M. L., in preparation), which would also affect the T helper cells and consequently the production of specific antibody.

Alternatively, it could be due to the production of a malarial mitogen stimulating lymphocytes nonspecifically, as recently shown by Greenwood and Vick (1975) in children infected with *Plasmodium falciparum*. The successful immunization with repeated injections of intact parasitized reticulocytes in contrast to the negative results obtained with parasitized mature erythrocytes supports the idea that "protective antigens" are released during the period of immunosuppression. Fluorescent or peroxidase-labeled antibodies were not seen to coat or penetrate infected mature red cells, confirming our previous results (Jerusalem, Weiss, and Poels 1971) and those of Hamburger and Kreier (1975) in rats. In contrast, however, the antibodies were able to penetrate in reticulocytes which were infected with three to four parasites (possibly leaking cells), supporting indirectly the selective availability of antigens during the suppressed period. The depression of the immunological responsiveness, perhaps as a result of T cell depletion and nonspecific recruitment of B

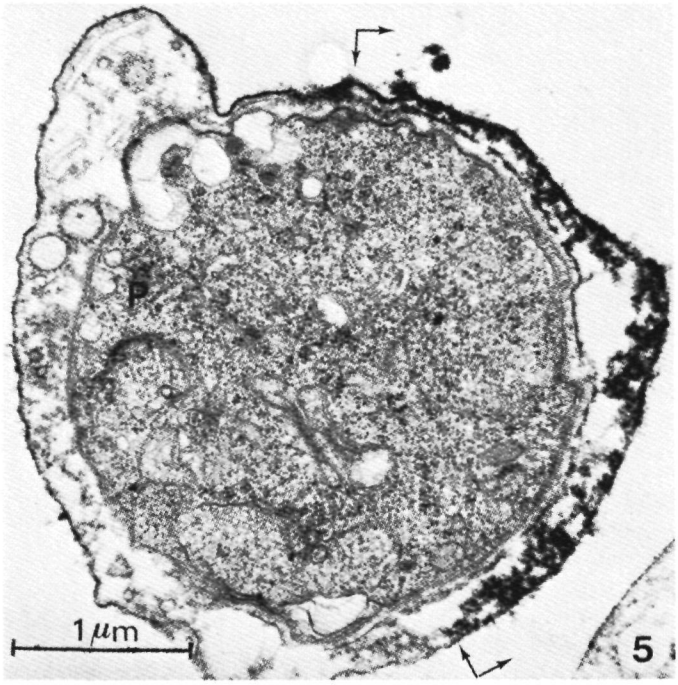

FIG. 5. Arrows indicate deposition of peroxidase-conjugated antibodies predominantly on the "leaky" side of a reticulocyte injected with *Plasmodium berghei*.

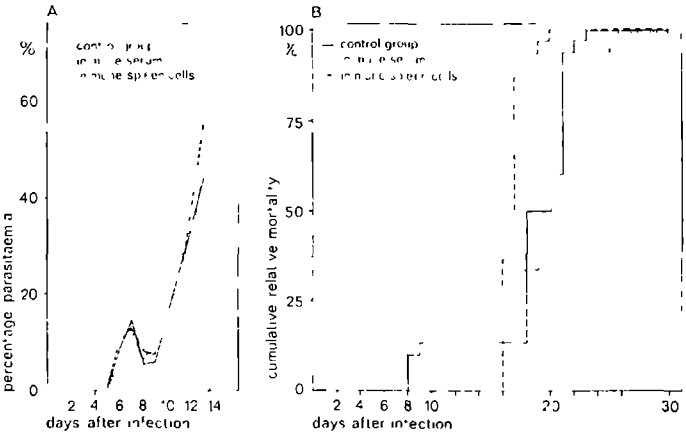

FIG. 6. The effect of transferred hyperimmune serum and spleen cells on the course of parasitemia and mortality in three groups of 40 BALB/c mice infected with 10^6 parasitized red blood cells (*Plasmodium berghei*). One group received 0.3 ml of hyperimmune serum intravenously on Day 5 and the second group was injected intravenously with 5×10^7 hyperimmune spleen cells. Graph A shows parasitemia, while B depicts the cumulative mortality.

cells by a mitogen, might have important consequences for the reaction to the malarial antigens released during that period. Antigens available during the period before the depression had low antigenicity, as judged from the weak response of splenic lymphatic tissue upon stimulation with parasitized oxyphilic cells. During the first week the parasite infects mostly mature oxyphilic RBC, and the schizonts appear to be largely sequestered in the deep tissue (Alger 1963). Both these factors may impede release of antigens.

On the other hand the release of antigens may be favored by an increasing osmotic fragility of erythrocytes as the infection progresses (Dunn 1969; Shen, Fleming, and Castle 1946), the effect being greater when most of the parasites (*Plasmodium lophurae* in ducks) were late segmenting forms, rather than young trophozoites (Herman 1969). Selective leakage of S antigens at a late stage of schizogony of *P. falciparum*-infected erythrocytes has been reported by Wilson and Bartholomew (1975). Cohen, Butcher, and Crandall (1969) have also referred to a slow release of *P. knowlesi* antigens *in vitro* during the initial phase of parasites growth and a more rapid release at schizogony. A selective availability of sufficient amounts of effective parasitic antigens might be basic for the differences in susceptibility to the action of antibodies on the various stages of the blood cycle, as reported for other plasmodial infections (Diggs and Osler 1975, Brown, Brown, Trigg, Phillips, and Hills 1970). The quantity and timing of availability of "protective" antigens, and probably also of B cell mitogens, to the host might be of crucial importance to the building up of protective immunity and are possibly basic to the interpretation of different results in different host–parasite combinations. For example, when lymphocytes are first exposed to antigens and then to B cell mitogens, the specific response will be enhanced, whereas the reverse sequence will result in depressed responsiveness (Andersson, Sjoberg, and Moller 1972a, b; Dutton 1975).

The second aspect of these experiments to be discussed concerns the transferability of immunity with either serum and/or

spleen cells. Although resistance to *P. berghei* in rats has been transferred with serum, the effect has always been of limited duration (Brown 1969; Brown and Phillips 1974; Diggs and Ossler 1969) and only small inocula were neutralized in rats, but not in mice (Golenser, Spira, and Zuckerman 1975; Briggs, Wellde, and Sadun 1966). Immune spleen cells and hyperimmune serum, raised in CBA mice against the self-limiting *P. yoelii*-protected intact recipients against 10^4 parasitized erythrocytes (Jayawardena, Targett, Leuchars, Carter, Doenhof, and Davies 1975). Our attempts to suppress an acute *P. berghei* infection in BALB/c mice with the aid of immune spleen cells and/or hyperimmune serum failed completely, though mortality was delayed by hyperimmune serum. Apparently only a limited number of the asynchronously growing parasites are available for reaction with inhibitory antibodies, in agreement with the fluorescence test. Protective immunity to *P. berghei* in rats was conferred by spleen cell suspensions (Stechschulte 1969; Phillips 1970; Phillips and Jones 1972), and Brown (1971, 1974) reported that T lymphocyte suspensions free of immunoglobulin-secreting B cells transferred protective immunity in rats. In the *P. yoelii*–CBA mice combination, Jayawardena, Targett, Levchars, Carter, Doenhof, and Davies (1975) showed that non-T cells were the effector cells in the transfer of protective immunity.

In our hands, the spleen cells from *P. berghei* immune BALB/c mice were not capable of transferring immunity to syngeneic infected recipients. Several reasons may be responsible for the ineffectiveness of spleen cell transfers in acutely infected mice. First, the efficiency of transferred RRBC-immune spleen cells to normal recipients was very low with respect to the production of antibodies or the propagation of B memory cells and agrees with estimates of effective homing of B memory cells to the spleen in the order of 1% (Askonas and Williamson 1972). Second, the availability of parasites to the immune defence system might be limited as discussed above. Third, generating a T cell-mediated immunity requires the cooperation between nonproliferating initiator T lymphocytes (ITL) and another T cell type which becomes recruited (RTL) to develop and proliferate in immunospecific effector and memory lymphocytes. These recruited T lymphocytes (RTL) are activated by the double signal of syngeneic ITL plus immunogen probably stored in macrophages (Cohen and Livnat 1976). The administration of malaria-sensitized ITL and RTL in acute *P. berghei*-infected mice will not be effective, as in these immunosuppressed animals the T cell function is apparently blocked (acute thymus involution and T cell depletion) thus preventing either the proliferation of the newly introduced RTL, or the recruitment of new immunospecific RTL by the injected sensitized initiator T lymphocytes.

However, we were able to confer protective immunity to *P. berghei* in noninfected normal mice by multiple injections of immune spleen cells (not contaminated with viable parasites) during a period of 4–6 weeks prior to challenge. These positive results may have been due to an uninhibited proliferation of sufficient T lymphocytes or to immunization with antigens retained in or on the immune spleen cells Kontiainen and Mitchison 1975). Alternatively, the transferred immune spleen cells might have released complexes of malarial antigens and antibodies which may have stimulated sufficient macrophage-like cells to secrete a nonspecific "protective factor" which interacts either with the parasite directly or with an infected cell, as has recently been suggested by Clark, Allison, and Cox (1976). The apparently contradictory results in the three host parasite combinations (rat–*P. berghei;* mouse–*P. yoelii,* and mouse–*P.*

berghei) concerning the variable degrees of immunosuppression and the transferability of protective immunity might depend upon which effector mechanism is selected by the available parasitic antigens as the most efficient system to "neutralize" the infection.

In summary, evidence has been presented that "protective antigens" are released during the course of *P. berghei* infection, probably by growing stages contained within polychromatophilic erythrocytes and at a time when the immune responsiveness is severely disturbed, thus preventing an effective response. This disturbance of the immune response may be the result of a combination of at least three factors: (a) severe depletion of T cells; (b) nonspecific recruitment of B cells by a mitogenic factor; and (c) consequent disruption of interaction with macrophages.

Acknowledgment

We wish to thank Professor C. Jerusalem for his help throughout this study. Dr. Margareth L. Weiss for useful suggestions and valuable advice during preparation of the manuscript, and Dr. P. Jap and co-workers for their help with the histological investigations. We acknowledge the assistance of Mrs V. van der Sterren and of Mr. G. Poelen. This study was supported by a grant of the World Health Organization and by the International Collaborative Research Project on Malarial Immunity and Immunopathology (I C R P./MII) supported by the Government of the Netherlands.

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CHAPTER 6

PLASMODIUM BERGHEI: FORMATION OF SECONDARY IMMUNE COMPLEXES IN HYPERIMMUNE MICE

LAMBERT G. POELS, CATHERINE C. VAN NIEKERK, AND LEO PENNINGS

Department of Cytology and Histology, Medical Faculty,
Geert Grooteplein Noord 21,
University of Nijmegen, Nijmegen, The Netherlands

AND

JACQUES AGTERBERG AND EVERT H. VAN ELVEN

The Central Laboratory of the Netherlands Red Cross Blood
Transfusion Service, Amsterdam, The Netherlands,
and Laboratory of Experimental and Clinical Immunology,
University of Amsterdam, Amsterdam, The Netherlands

(Accepted 16 May 1977)

Experimental Parasitology 43, in press.

ABSTRACT

BALB/c mice infected with the lethal strain Plasmodium berghei (K 173) and hyperimmune BALB/c mice which had been recovered from the infection after chloroquine therapy were used to study the formation of immune complexes in glomeruli of the kidney and in plasma, and their role in the induction of nephropathies, using electron microscopy, immunofluorescence immunoperoxidase and immunodiffusion techniques.

Electron microscopical studies revealed comparable ultrastructural alterations in the glomeruli of infected and hyperimmune mice. Immune complexes in the glomeruli of infected mice were found to consist of immunoglobulins IgM, IgA and IgG, complement factor, and plasmodial antigens. On the other hand, precipitating plasmodial antibodies were hardly detectable in plasma of infected mice, while plasmodial antigens were present. An increased urinary protein content was recorded in infected mice. The sera were negative in the rheumatoid factor assay.

Eradication of the parasitemia by chloroquine therapy caused a gradual disappearance of plasmodial antigens from the glomerular immune complexes and from plasma within three weeks. The glomeruli, however, remained strongly positive for immunoglobulins and complement.

Plasma and glomeruli of hyperimmune mice which had been free of parasites up to ten weeks remained strongly positive for

immunoglobulins and complement, and completely negative for plasmodial antigens. No increased urinary protein content was recorded. The sera were negative for rheumatoid factor.

Intravenous injections of hyperimmune serum into normal control mice and into infected athymic nude mice caused in both cases a deposition of immune complexes in the glomeruli, being negative for plasmodial antigens in the control recipients, and positive in the infected nude group.

These findings provide evidence that Plasmodium berghei infection in BALB/c mice caused a non-transient deposition of immune complexes of two different types: the first type in which plasmodial antigens could be demonstrated during an acute infection; a second type in which plasmodial antigens could not be demonstrated and which occurred in the plasma of recovered hyperimmune mice.

INDEX DESCRIPTORS: Plasmodium berghei; Parasitic protozoa; Malaria; BALB/c mice; Immune complexes; Acute and chronic nephritis; Glomerular ultrastructure; Plasmodial antigens; immunofluorescence and immunoperoxidase; Precipitating antibodies; Complement; Rheumatoid factor.

INTRODUCTION

Plasmodium berghei infection in BALB/c mice is lethal to all mice within 30 days after inoculation. The infection causes a severe depression of the immune responsiveness to sheep red blood cells and xenogeneic skin grafts (Sengers, Jerusalem and Doesburgh 1971). During the period of immunodepression considerably elevated levels of non-specific antibodies, predominantly IgG were produced, while specific antiplasmodial antibodies were hardly detectable with the indirect hemagglutination test (IHA) and immunofluorescence technique (Poels and van Niekerk 1977). Varying degrees of immunosuppression and production of specific antibodies are effected by different plasmodial strains in different hosts (Jayawardena, Targett, Leuchars, Carter, Doenhof and Davies 1975). Diverse responses find expression also in the nephropathy associated with the deposition of immune complexes in renal glomeruli (see reviews by Adeniyi and Akingkugbe 1972, Houba 1975 and Weise, Ehrich and Voller 1976). At least two types of immunologically determined nephropathies are known; acute transient immune-complex glomerulonephritis with reversible lesions, typical of P. falciparum infections in man, and chronic (progressive) immune-complex glomerulonephritis with irreversible glomerular lesions, characteristic of quartan infections in man. In the latter, antimalarial therapy has no effect. Immunohistological investigations in malarial rodents have been reported by Ehrich and Voller (1972), Suzuki (1974), Boonpucknavig, Boonpucknavig and Bhamarapravati (1972, 1973), Weise, Ehrich and Weise (1972) and Weise, Konitz, Weise and Ehrich (1973) showing that the ultra-

structural glomerular changes were specific for the species of Plasmodium. Similarly the glomerular alternations and proteinuria were found to be transient and reversible in self-limiting P. yoelii infections, while the lethal P. berghei caused more severe glomerular lesions (Weise, Ehrich and Voller 1976). The barely detectable antiplasmodial antibodies in the plasma of mice infected with P. berghei (Poels and van Niekerk 1977), and the reported deposition of immune complexes in renal tissue (Boonpucknavig, Boonpucknavig and Bhamarapravati 1972) prompted us to investigate the formation of immune complexes and their role in nephropathies in mice infected with P. berghei in comparison with chloroquine cured and hyperimmune mice which had high titers of antiplasmodial antibodies (Poels and van Niekerk 1977). The degree of renal damage was determined by studying the ultrastructural glomerular changes and the proteinuria. The requirements for formation of "proper" immune complexes in plasma and glomeruli were studied in three models: 1) infected normal mice, expected to be positive for circulating plasmodial antigens and antibodies, 2) hyperimmune mice, expected to be positive only for specific antibodies, and 3) infected, congenitally athymic mice, expected to be positive only for circulating plasmodial antigens as the thymus-dependent production of antibodies is severely impaired (Pantelouris 1973).

MATERIALS AND METHODS

Vertebrate host and parasite.

Specific pathogen free inbred BALB/c mice (+/+), 10 to 20 weeks of age, were maintained on Laboratory Standard Diet (Hope Farms Ltd.) and water ad libitum.
Eight-week-old congenitally athymic (nude) mice ($^{-}$/-), backcrossed at least 25 generations on a BALB/c background were obtained from TNO (Zeist, The Netherlands); they were specific pathogen-free, and were kept separated from the other mice, in cages at 28° C, and maintained on the same diet. Plasmodium berghei berghei (strain K 173) was routinely passaged by intraperitoneal inoculation of 10^5 infected red blood cells (p. RBC) in (+/+) BALB/c mice. Parasitemias were determined in blood smears prepared from tail blood and stained with May-GrUnwald/Giemsa. Parasitemia and reticulocytosis had reached a level of approximately 60% on day 14 of infection (Poels and van Niekerk 1977). The course of parasitemia and reticulocytosis in (+/+) and ($^{-}$/-) mice was comparable, while the mortality in nude mice was usually delayed (MS in preparation).

To cure the infection 300 mg chloroquine/L was added to the drinking water for one week.

A standard procedure for immunization of mice to the otherwise lethal P. berghei was used as described previously (Poels, van Niekerk, Franken and van Elven 1977). Briefly, adult mice (+/+) were infected with 10^5 p.RBC. The parasitemia was eradicated at day 14 with chloroquine therapy. All surviving mice (85%) were found to be immune to challenge on day 43, and subsequent boosting

with 10^7 p. RBC every 2 weeks. Hyperimmune mice remained free of parasites as judged from blood smears and negative isodiagnosis.

Preparation of plasmodial antigens.

Antigens of P. berghei were prepared as described by Lunde and Powers (1976). Briefly, heparinized blood was obtained from BALB/c mice 14 days post-infection. The cells were washed four times in physiological saline (0,14 M NaCl). A final 50% suspension was stored at -25° C overnight and thawed the next day at 37° C for 30 minutes. The preparation was centrifuged at 10 000 g for 30 minutes. The supernatant lysate was used as soluble "standard" antigen and stored in aliquots of 0.5 ml at -25° C. A similar antigen was prepared from blood of nude BALB/c mice ($^-$/-) 14 days post-infection, and from non-infected blood which served as control "standard" antigen preparation.

Antisera and conjugates.

Rabbit anti-mouse IgG-FITC (RAM-FITC) conjugate was prepared as described previously (Jerusalem, Weiss and Poels 1971). Alternatively goat anti-mouse IgG-FITC (GAM-FITC) from Nordic Pharmaceuticals (Tilburg, The Netherlands) was used in the recommended dilution. IgA and IgM were detected by using RAM/IgA and RAM/IgM (Nordic) respectively, in combination with Swine anti-rabbit serum IgG-FITC (SwAR-FITC) (Dako, Denmark).

Complement factors C_3 and C_4 were detected with rabbit-anti mouse $C_{3,4}$ obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, in combination with SwAR-FITC.

Immunoglobulins from mice hyperimmune to malaria were isolated and conjugated with horse radish peroxidase (HRP, Boehringer, Mannheim, No. 10.80.90) as described by Boorsma and Streefkerk (1976), using the two step glutaraldehyde activation technique. The procedure yields a HRP conjugated monomer IgG that is separated completely from monomer IgG.

Immunohistological techniques.

The immunofluorescence technique was used to study the presence of immunoglobulins IgM, IgA, IgG and complement factors in cryostat sections of snap-frozen kidneys by the method of Thé and Feltkamp-Vroom (1970). Triplicate sections of 5 to 15 mice per group were examined independently by two or three persons for localization and granularity of the fluorescence. The fluorescence was scored arbitrarily from negative (0) to (4+) according to the degree of intensity and extension of the fluorescence.

The immunoperoxidase technique was used to detect plasmodial antigens in renal tissue. Cryostat sections of the kidney were pretreated in a similar fashion as for the immunofluorescence technique. After incubation with peroxidase conjugated anti-malarial IgG the sections were washed in PBS, stained with 3,3'-diaminobenzidine and H_2O_2 (Graham and Karnowsky 1966), washed in PBS, and fixed in

OsO_4 (Millonig 1961) for 1-4 minutes. When necessary, endogenous pseudoperoxidase activity was inhibited, as described by Streefkerk (1972).

Electron microscopy of renal tissue

Cortical portions of the kidneys of control and experimental mice were fixed in 2% osmium tetroxide (Pallade 1952), dehydrated in ethanol and embedded in Epon (Luft 1961). Ultrathin sections of at least 5 glomeruli of each kidney were double stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963), and examined with a Philips EM 300 electron microscope.

Immunodiffusion test.

Glass slides were covered with 0.85% agarose (Boehringer, Mannheim) buffered in Gelman's High Resolution Buffer (Gelman, Michigan) pH 8.2. Wells of 8 mm diameter were punched at intervals of 3 mm. The maximum volume per well was 70 µl. All sera and plasmas used in these tests were "cleared" of particles and parasitic contamination by an additional centrifugation at 10 000 g for 15 minutes. The supernatant was then filtered through a 0.45 µm Millipore filter and stored in small aliquots at - 25°C.

Rheumatoid factor (RF)

The rheumatoid factor was assayed with a modified Rose-Waaler hemagglutination system as described by Feltkamp (1966) and van Loghem-Langereis (1952). The determinations were done in duplicates of at least 8 mice per sample.

Protein determination in urine.

Semiquantitative protein content of the urine of individual mice was tested twice a day by commercial paperstrip method (Boehringer, Mannheim). The samples were scored from negative (0) (< 25 mg/100 ml) to (3+), (500 mg/100 ml). Eight mice per group (infected, hyper-immune and control mice) were tested.

RESULTS

1. Immune complexes in mice during infection with P. berghei, and after chloroquine treatment.

At the times post-infection indicated in Table 1, cryostat sections of the kidneys of mice infected with P. berghei were examined for the presence of immunoglobulins and complement by immunofluorescence, while localization of plasmodial antigens was assayed by the immuno-peroxidase technique. An increasing quantity of immune complexes was deposited as fine granules around capillaries and in the mesangial matrix of the glomeruli during a progressing infection (Table 1, group A). The immune complexes were positive for immunoglobulins IgM, IgA and IgG, for complement factors $C_{3/4}$, and for plasmodial antigens. An increasing fluorescence for immunoglobulins and complement was noticed in the interstitial tissue; however, a semiquantitative evaluation was difficult.

Electron microscopy of glomeruli of mice 14 days post-infection revealed frequently coarse electron dense subepithelial precipitates, occasionally small subendothelial aggregates along the sometimes irregularly thickened capillary basement membrane, and electron-dense

mesangial deposits in the glomeruli. The endothelial cells of the glomerular capillaries were swollen occasionally. Evident proliferation of the mesangial area was observed in only one glomerulus. Local splitting of the basement membrane occurred. Fusion of foot processes of the podocytes occurred very frequently and was quite extensive over long areas of the capillary basement membrane. Some glomerular and interstitial capillaries contained mononuclear cells with electron-dense, cristalloid inclusions of so called "malaria pigment". Occasionally plasma cells were found interstitially.

The gel immunodiffusion assay (Figure 1) revealed that plasma of (+/+) mice 14 days post-infection contained three antigens being identical to those found in infected (-/-) mice. The identity between circulating antigens and "standard" antigens was not always complete, and second not all antigens present in the "standard" antigen preparations were present in plasma of infected mice.

Occasionally, antiplasmodial antibodies were detected in some samples of infected (+/+) plasma concentrated two fold. Control "standard" antigen preparations and control plasma from non-infected red blood cells never yielded any precipitation line with any of plasma samples tested.

Complement factors C_3 and C_4 were found in control plasma and plasma obtained from (+/+) mice 7 days post-infection. The C_4 line, however, was very weak in plasma of (+/+) mice 14 days post-infection (Figure 2).

All sera of control and infected mice were found to be negative in the rheumatoid factor assay, as shown in Table 1. A protein range from 1+ to 3+ albuminuria was recorded in malarious urine on days 8 and 14 post-infection (Table 1). The degree of proteinuria, however, could not be correlated with the extent of parasitemia. Control urine was always negative.

After parasitemia was eradicated with chloroquine (Table 1, group B) the fluorescence for immunoglobulins and complement increased gradually, while the localization and granularity was similar to that in glomeruli of mice 14 days after infection. The immunoperoxidase reaction for plasmodial antigens decreased gradually. A very slight amount of parasitic antigens was detectable on day 42 at which time the animals had been free of parasites for three weeks. Plasmodial antigens were not detectable in the glomerular deposits on day 70.

Plasmodial antigens were not detected in plasma of immune mice (day 42 after a cured infection).

Antiplasmodial antibodies were present in immune plasma, although not all precipitation lines were found to be identical with the lines formed between hyperimmune serum and "standard" antigen (Figure 1).

Anti-$C_{3/4}$ serum yielded two precipitation lines with immune plasma, being identical with the lines from control plasma (data not shown).

The rheumatoid factor test was negative for immune plasma (Table 1), and no proteinuria was noted in cured animals (day 42 and 70, Table 1).

2. Immune complexes in hyperimmune mice.

A group of (+/+) BALB/c mice was immunized to P. berghei by the chloroquine procedure described in Methods. The mice were

boosted every two weeks with 10^7 infected RBC over a ten week period. Incidental survival of a parasite was prevented by chloroquine treatment for one week started at the time of the last booster. The renal glomeruli of frequently boosted hyperimmune boosted hyperimmune mice (Table 1, group D) remained strongly positive for immunoglobulins and complement as determined up to 8 weeks after the last booster. The fluorescence was localized in fine granules around the capillary walls and was very intense in the mesangial areas. Plasmodial antigens, however, were never detectable in the renal precipitates of hyperimmune mice. In order to investigate whether the immunoglobulins present in glomeruli of hyperimmune mice had any specificity to plasmodial antigens, kidney sections of hyperimmune mice were incubated with plasmodial "standard" antigen solutions at different concentrations (1/1 to 1/50), washed and subsequently tested for bound plasmodial antigens with the immunoperoxidase technique. The results were negative for all concentrations of plasmodial antigens tested.

Electron microscopy of glomeruli of hyperimmune mice revealed that in some glomeruli electron-dense precipitates were located predominantly subepithelial and subendothelial along the capillary basement membrane (Figure 3), while in other glomeruli electron-dense precipitates were found predominantly in the mesangial areas (Figure 4) in which case proliferation of the mesangium was evident. Often, the capillary endothelial cells were swollen. The glomerular basement membrane was irregularly thickened and swollen locally (Figure 5).

Fusion of foot processes of the podocytes was usually less than in infected mice and restricted locally (Figure 4). Mononuclear cells with cristalloid inclusions were usually not observed in capillaries and blood vessels.

Hyperimmune plasma yielded several precipitation lines with (+/+) and (-/-) plasmodial "standard" antigens (Figure 1). Precipitation lines were never formed between hyperimmune plasma and control "standard" antigens or control plasma.

Anti-C serum yielded two precipitation lines (C_3 and C_4) with hyperimmune plasma (Figure 2).

Hyperimmune sera were negative in the rheumatoid factor assay (Table 1), and no proteinuria was recorded in hyperimmune mice (Table 1).

3. Immune complexes in infected nude mice (-/-).

Infected nude mice (Table 1, group C) never showed any deposition of immune complexes in the kidney.

Plasma of nude mice 14 days post-infection contained three plasmodial antigens which showed identity with the antigens present in malarial (+/+) plasma and no, or partial identity with nude "standard" antigens (Figure 1). The nude "standard" antigen and the (+/+) "standard" antigen appeared to be identical in tests with hyperimmune plasma (not shown in Figure 1). The malarious nude plasma never contained precipitating antibodies to plasmodial antigens.

Anti - $C_{3/4}$ serum yielded a clear line for C_3 and a very weak line for C_4 (Figure 2).

The infected nude plasma was negative in the RF assay. Proteinuria was not determined.

4. Transfer of plasma of infected and hyperimmune mice.

To investigate whether immune complexes were circulating in plasma of infected and hyperimmune mice plasma transfer studies were performed. The results (Table 2) show that three daily i v. injections of 0.3 ml plasma obtained from (+/+) mice on the 14th day post infection or from hyperimmune mice caused an evident deposition of immune complexes in the glomeruli of non-infected recipients. The precipitates were positive for IgG, complement and plasmodial antigens when malarious plasma had been transferred, but negative for plasmodial antigens when hyperimmune plasma had been used. Transfer of malarious plasma from nude mice did not cause any deposition of immune complexes in non-infected recipients. In all cases the immunofluorescence reaction for complement was weaker than for IgG. However, when nude mice, 14 days post-infection were injected with three daily doses of 0.3 ml hyperimmune plasma, the deposited immune complexes were strongly positive for plasmodial antigens, in contrast to the non-infected (+/+) mice, as determined up to 7 days after the last injection of hyperimmune plasma.

DISCUSSION

In the present study we have investigated the formation of immune complexes and their subsequent deposition in renal glomeruli of mice which suffered a progressive P. berghei infection and of hyperimmune mice in which the infection was cured with chloroquine. A comparative study was done on the nude mouse model.

Immune complexes were deposited in renal glomeruli of acutely infected (+/+) mice and hyperimmune mice but not in the glomeruli of infected nude (-/-) mice. Pathological ultrastructural alterations were found in the glomeruli of the first two groups. The glomerular immune complexes found in acutely infected (+/+) mice contained immunoglobulins IgM, IgA and IgG, complement and plasmodial antigens. The ultrastructural glomerular changes and the increased proteinuria observed in our infected (+/+) BALB/c mice confirm the findings of Boonpucknavig, Boonpucknavig and Bhamarapravati (1972, 1973), and Suzuki (1974) and indicate an acute glomerulonephritis, as also described for other models (Houba 1975; Weise, Ehrich and Voller 1976). The immune complexes found in hyperimmune mice contained immunoglobulins and complement.

Plasmodial antigens, however, were not detected in the glomeruli, and no increased protein excretion in the urine was recorded. This could indicate that plasmodial antigens had disappeared gradually from the glomerular precipitates after chloroquine treatment or alternatively, that plasmodial antigens were masked by excess of specific antibodies as shown to be present in immune and hyperimmune plasma. Our results provide several arguments in favour of the first possibility. The hyperimmune mice had been free of living parasites for a very prolonged period, due to chloroquine treatment from day 14 to 21, and once more at the time of the tenth (last) booster. No plasmodial antigens were detected in hyperimmune plasma in the immunodiffusion test. Transfer of hyperimmune plasma to non-infected recipients caused considerable deposition of immune complexes which

were negative for plasmodial antigens, while administration of hyperimmune plasma to infected nude mice caused deposition of immune complexes which were strongly positive for plasmodial antigens, reflecting at the same time high levels of free circulating plasmodial antigens in the infected nude mice. The immunoglobulins in the glomerular immune complexes of hyperimmune mice did not bind additional plasmodial antigens when the kidney sections were incubated with plasmodial "standard" antigens, indicating that a supposed excess of antibodies in the immune complexes did not have any specificity to plasmodial antigens. It seems reasonable therefore to suggest that the immune complexes circulating in hyperimmune mice and deposited in the glomeruli contain immunoglobulins with no specificity to plasmodial antigens. One can only speculate on what kind of unidentified antigens may be involved in the formation of immune complexes in hyperimmune mice. Although the rheumatoidlike factor (RF) test was negative for malarious as well as hyperimmune plasma and no precipitation lines were formed between control plasma and hyperimmune or malarious plasma in the immunodiffusion test, the formation of antibodies with specificity for autologous immunoglobulins modified by complex formation with parasitic haptens is not entirely excluded as mopping up of antigen and/or antibody, and subsequent clearance by deposition of such complexes in the kidney appears a possibility. The alternative release of autologous tissue antigens, due to renal or blood vascular damage and subsequent formation of antibodies to these antigens, is unlikely because of the absence of a linear fluorescence pattern. The formation of a secondary immune complex after chloroquine treatment, possibly as an autoimmune process, would explain earlier findings that immunoglobulin

levels in plasma of hyperimmune mice remained high, which is also in accordance with a sustained plasma cellular reaction and active germinal centers observed in the spleen (Poels and van Niekerk 1977). Our findings differ from those observed in the self-limiting P. yoelii model described by Ehrich and Voller (1972), and by Weise, Konitz, Weise and Ehrich (1973) in which a transient deposition only of immune complexes was reported, although a persistent deposition of immune complexes was recorded recently in the same model by George, Parbtani and Cameron (1976). Our findings differ also from the data obtained from P. malariae patients (Houba 1975; Weise, Ehrich and Voller 1976) in which the progressive deposition of immune complexes in the kidney after antimalarial therapy was accompanied by a persistent clinical nephrosis in contrast to our hyperimmune mice.

The size and/or composition of immune complexes play a crucial role in the induction of a proteinuria (Dixon 1971). The presence of plasmodial antigens in the glomerular immune complexes in infected mice and the absence in hyperimmune mice, or the antigen-antibody balance in the immune complexes being in favour of plasmodial antigens in malarious mice and in favour of the antibodies in recovering mice, might possibly be responsible for the difference in pathogenicity of the deposited immune complexes.

The role of complement in inducing glomerular lesions and subsequent proteinuria seems minor in P. berghei infection for several reasons. The immunofluorescence for complement in glomeruli of hyperimmune mice was much stronger than in acutely infected mice, but did not seem to induce proteinuria. The decreased level of C_4 in plasma

of infected (+/+) mice was probably not due to complement consumption, as the C_4 level was similarly reduced in infected nude mice 14 days after infection in the absence of antiplasmodial antibodies or immune complexes in these mice. In contrast to a frequently reported reduced C_3 level (Weise, Ehrich and Voller 1976) the reduced C_4 level and subsequent less binding in immune complexes of infected mice might be explained by a decreased synthesis of C_4 in macrophages (Lachman 1975) rather than excess consumption of complement by immune complexes.

The deposition of immune complexes might have some important consequence for the level and selection of both circulating plasmodial antigens and anti-plasmodial antibodies in the plasma of infected mice. The identity of circulating plasmodial antigens in infected normal and nude mice makes it unlikely that any antigens released from parasites in vivo were selectively altered or removed by specific antibodies, as nude mice did not produce antiplasmodial antibodies (also Weiss, in press). The "standard" antigen preparations (+/+ and -/-) contained antigens which were identical to the antigens present in the plasma but contained also antigens with partial identity and non-identity. The freeze thawing procedure for preparation of our "standard" antigens might have caused some artificial desintegration or realignment of antigens.

In summary, evidence has been presented that in mice acutely infected with P. berghei, free and complexed plasmodial antigens circulate in the blood mopping up all free antibodies, which become subsequently deposited in the renal glomeruli causing pathological structural alterations and proteinuria. In hyper-immune mice no plasmodial antigens, either free or complexed, were detected in plasma and glomeruli, while specific anti-plasmodial antibodies were circulating freely. However, secondary complexes of immunoglobulins with an as yet unidentified antigenic factor were present in the plasma and deposited in the glomeruli. Possibly, such an absence of malarial antigens in precipitated renal complexes might be the reason for the ineffectiveness of antimalarial chemotherapy in cases of malaria induced chronic nephropathies. The mechanism of the formation of such secondary complexes and its characterization is under current investigation.

ACKNOWLEDGEMENT

We wish to thank Professor Dr. C. Jerusalem for his help throughout this study. We are much obliged to Dr. P. Jap and coworkers for their help with the histological and electron-microscopical investigations. We thank Dr. Margareth L. Weiss for useful suggestions and valuable advice during preparation of the manuscript. We acknowledge the assistance of Mrs. V. van der Sterren, and of Mr. J. Reitsma and collaborators (Central Animal Laboratory). We are indebted to Miss Annelies Schreurs and Mrs. Mary Scholten for typing the manuscript.
This study was supported by a grant of the World Health Organization and by the International Collaboration Research Project on Malaria Immunity and Immunopathology (ICRP/MII) supported by the Dutch Government.

Table 1
Immune complexes in glomeruli in *Plasmodium berghei* infected (+/+ and ⁻/-) mice, and in immune and hyperimmune mice.

	Days after infection	IMMUNOHISTOCHEMICAL REACTIONS ON RENAL GLOMERULI					SEROLOGY	UROLOGY
		Immunofluorescence				Immuno-peroxidase		
		IgM	IgA	IgG	$C_{3/4}$	malarial antigens	rheumatoid factor	protein-uria
Group A								
BALB/c mice	0	0	0	0	0	0[a]	0	0
infected (+/+)	3	2+	1+	1+	0	ND	ND	ND
(untreated)	8	2+	2+	2+	1+	ND	ND	+/++
	14	3+	3+	3+	2+	4+	0	+++
	21	3+	3+	3+	2+	4+	0	+++
Group B								
BALB/c mice	21	4+	4+	4+	2+	4+	ND	ND
infected (+/+)	28	4+	4+	4+	3+	ND	ND	ND
chloroquine	42	4+	4+	4+	4+	1+	0	0
therapy from	70	3+	3+	3+	4+	0	0	0
day 14-21	0[b]	0	0	0	0	0	ND	0
Group C								
BALB/c mice	0	0	0	0	0	0	0	ND
infected (⁻/-)	8	0	0	0	0	0	ND	ND
(untreated)	14	0	0	0	0	0	0	ND
Group D[c]								
BALB/c mice hyperimmune (+/+)								
— 1 week after booster		4+	4+	4+	4+	0	0	0
— 8 weeks after booster		4+	4+	4+	4+	0	0	0

a) ND = not done

b) Chloroquine control: non-infected (+/+) mice examined 10 days after a week of chloroquine treatment.

c) Chloroquine treatment was given for one week at the time of the last booster.

Table 2

Composition of immune complexes in glomeruli after injection of malarious (Plasmodium berghei) plasma (+/+ and -/-) and hyperimmune plasma.

mice plasma donors	mice recipients[a]	Number of i.v. injections 0.3 ml/shot	Immuno-fluorescence IgG	Immuno-fluorescence C[b]	Immuno-peroxidase plasmodial antigens
normal uninfected control (+/+)	normal (+/+)	1	0	0	0
	normal (+/+)	3	0	0	0
14 day infected (+/+)	normal (+/+)	1	1+	1+	1+
	normal (+/+)	3	2+	1+	1+
14 day infected (-/-)	normal (+/+)	1	0	0	0
	normal (+/+)	3	0	0	0
hyperimmune	normal (+/+)	1	1+	1+	0
	normal (+/+)	3	3+	1+	0
hyperimmune	14 day infected (-/-)	3[c]	3+	1+	3+
		3[d]	3+	1+	3+

a) kidneys of at least five recipients were examined 24 hr after the last injection of plasma.

b) complement

c) glomeruli were investigated 1 day after the third injection of hyperimmune plasma.

d) glomeruli were investigated 7 days after the third injection of hyperimmune plasma.

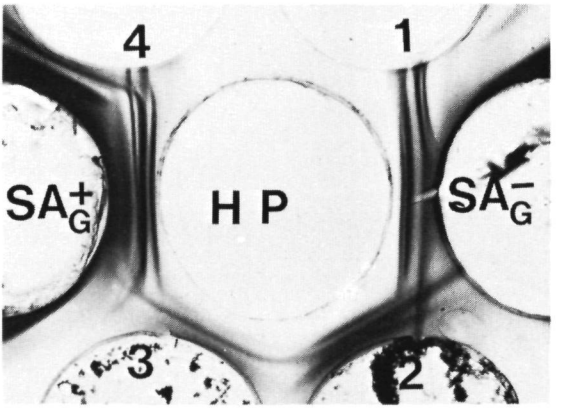

FIG. 1. Plasmodial antigens and antiplasmodial antibodies in plasma of mice infected with Plasmodium berghei and of hyperimmune mice. HP = hyperimmune plasma; SA^{+}_{G} and SA^{-}_{G} = "standard" antigen prepared from $^{+}/+$ and $^{-}/-$ mice 14 days post-infection, respectively; 1 = non-infected control plasma; 2 = plasma of nude mice ($^{-}/-$) 14 days post-infection; 3 = plasma (2x concentrated) of $^{+}/+$ mice 14 days post-infection; 4 = plasma of immune mice (before challenge).

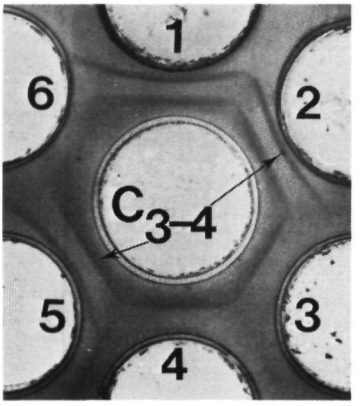

FIG. 2. Complement factor C_3 and C_4 in plasma of mice infected with Plasmodium berghei and of hyperimmune mice. C_{3-4} = rabbit anti-mouse C_{3-4} serum; 1 = non-infected control plasma from $^{+}/+$ or $^{-}/-$ mice; 2 = plasma of $^{+}/+$ mice 7 days post-infection; 3 = plasma of $^{+}/+$ mice 14 days post-infection; 4 = plasma of $^{+}/+$ mice 14 days post-infection; 5 = hyperimmune plasma; 6 = albumine solution (1%). The precipitation line between 5 and 4 represents plasmodial antigens and antiplasmodial antibodies.

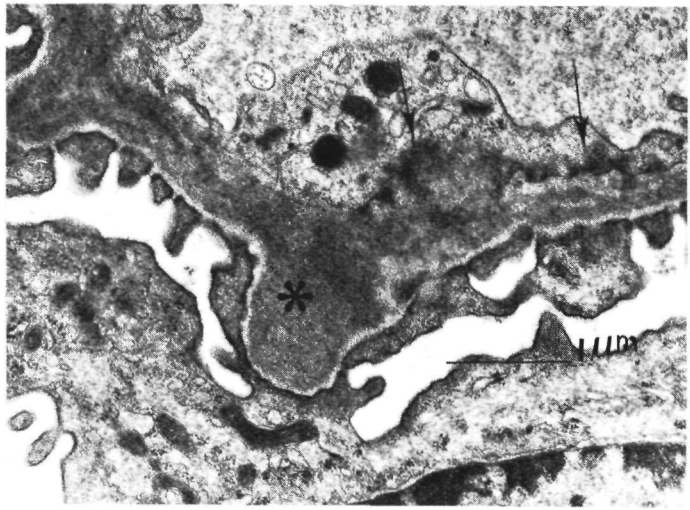

FIG. 3. Subendothelial deposits (arrows) on the glomerular basal membrane and humps (asterisk) in the GBM of a mouse hyperimmune to Plasmodium berghei.

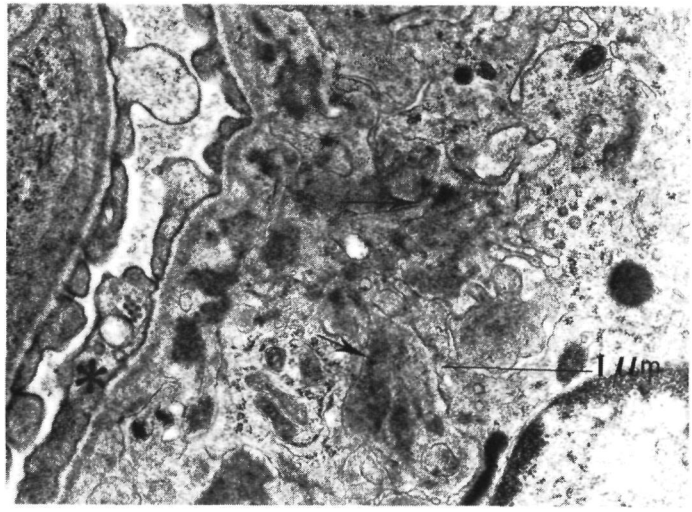

FIG. 4. Mesangial deposits (arrows) and fusion of podocytes (asterisk) in the glomerulus of a mouse hyperimmune to Plasmodium berghei.

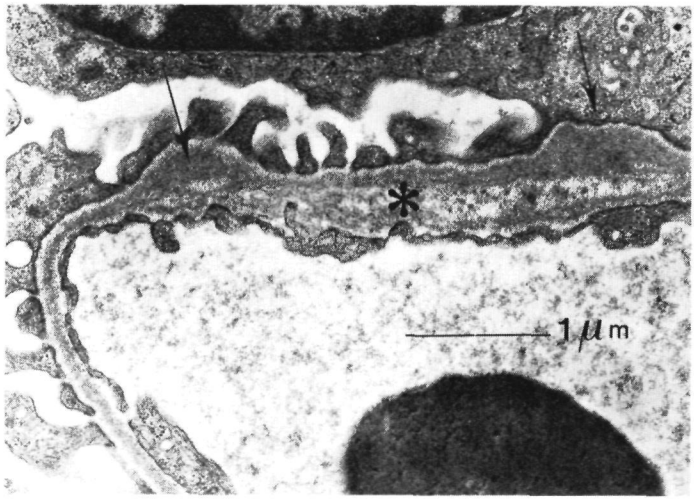

FIG. 5. Humps (arrows) and swollen GBM (asterisk) of a mouse hyperimmune to Plasmodium berghei.

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CHAPTER 7 GENERAL DISCUSSION

GENERAL DISCUSSION

Although the results of the investigations have been described and discussed in the preceding papers the relevance of some characteristics of a Plasmodium berghei infection in BALB/c mice, as summarized in Table 1, will be discussed in this chapter, in order to define an outline for research in the future.

First, it is stated, once again, that this infection is lethal to all mice within 30 days after infection; second, eradication of the parasitemia with chloroquine on day 14 of infection leads to the development of a protective immunity as outlined in Fig. 1. The immunity appears to be sterile. A "clinical" complication, however, might be the continuous formation and deposition of secondary immune complexes in the kidney in the absence of plasmodial antigens, suggesting an autoimmune process (Poels, van Niekerk, Pennings, Agterberg and van Elven 1977). This is currently under investigation.

Two questions can be considered:

I. How does the parasite escape the host defence mechanism in an acute infection

II. How is the parasite eliminated in immune animals

The outcome of an infection may depend on the balance between the amount of the infectious proliferating parasites, and on the other hand, the (enhanced or reduced) capacity of the host to resist the infection.

I. How does the parasite escape the host defence mechanism in an acute infection

Considering the immune status of acutely infected mice it appears that the immune responsiveness to heterologous erythrocytes is initially somewhat increased but, when the infection progresses, becomes suppressed completely. The character of immunosuppression and tolerance phenomenon has been discussed in a preceding paper (Poels and van Niekerk 1977). There is some evidence that the remarkable thymic involution in acutely infected mice is initially caused by an accelerated migration of thymocytes from the thymic cortex via the medulla to the lymphatics comparable to a permanent ductus thoracicus drainage (Jerusalem, personal communication). The initial increased recruitment of T lymphocytes might be related to the nonspecific enhanced differentiation or Plaque Forming Cells (PFC) (Poels and van Niekerk 1977) observed when the animals were primed with heterologous erythrocytes at the very beginning of the infection. This is in agreement with a model proposed by Waldman and Munro (1975) stating that a T cell factor produced by antigenic stimulation can act nonspecifically in facilitating the response of B cells to a non-cross-reacting antigen.

In a later phase of the infection the proliferation of cortical thymocytes is inhibited almost completely as shown by autoradiography using tritiated thymidine (Jerusalem and De Wit 1974). Both phenomena, thymocyte recruitment and the subsequent inhibition of proliferation, explain the implications of thymic

involution for the immune responsiveness to heterologous erythrocytes and possibly to parasitic antigens released either at the beginning of the infection or later during the acute phase.

Although the status of immunosuppression will render the infected mice more susceptible to other infections (Wedderburn 1970; Cox 1975) this does not imply a priori that the reactivity to plasmodial antigens is affected to the same extent. The production of nonspecific antibodies, for example, appears not to be blocked. Specific antiplasmodial antibodies, however, are hardly detectable in acutely infected mice (Poels and van Niekerk 1977). The detection of immune complexes containing antibodies, complement and parasitic antigens, circulating in the blood and subsequently deposited in the renal glomeruli proves that specific antibodies are produced during the acute phase (Poels, van Niekerk, Pennings, Agterberg and van Elven 1977). The formation of these immune complexes, their unknown circulation time, and half life of the glomerular deposits make it difficult to determine whether these specific antibodies were synthesized predominantly during the first seven days of infection when immunosuppression is still mild, or also in the later period of severe unresponsiveness to heterologous erythrocytes. Determination of the amounts of circulating immune complexes, currently investigated, might provide some additional information.

Our experiments have shown that during the period of severe immunosuppression parasitic antigens become available, which can evoke

a protective immunity after the mice have been cured with chloroquine (Poels, van Niekerk, Franken and van Elven 1977). These "protective" antigens are not, or not in sufficient quantities, available during the early stage of infection when the parasites have invaded mainly oxyphilic cells. Alternatively, the absence of an efficient immune response in a rapidly progressing infection possibly enables the parasites to expose a broad range of antigenic variants to which the host becomes immunized simultaneously after chloroquine therapy, suggesting a variant on the reported sequential development of a single new antigenic variant in chronic relapsing infections with Plasmodium knowlesi (Brown 1971; Brown and Hills 1974; 1976; Brown, Jarra and Hills 1976). The coincidence of immunosuppression with the selective release of "protective" antigens may partly explain why in this combination of P. berghei - BALB/c mice the infection can never be self-limiting (Poels, van Niekerk, Franken and van Elven 1977).

In addition to a selective release of "protective" antigens during the period of immunosuppression, a shortage of specific antiparasitic antibodies in acutely infected mice might be important for the outcome of the infection. The insufficient amount of antibodies could be due either to a suppressed synthesis or an ample production of parasitic antigens clearing the antibodies as soon as they are released. The role of antibodies, however, seems to be minor in eliminating the parasitemia in P. berghei infected mice, in contrast to some other host-parasite combinations. Passive transfer

of serum from hyperimmune donors has been shown to modify the course of infection in rats (Diggs and Osler 1969; Golenser, Spira and Zuckerman 1975; Brown and Philips 1974) and offers partial protection against human malaria (P. falciparum) (McGregor, Carrington and Cohen 1963). Protection was usually partial, it decreased the duration or magnitude of parasitemia and/or improved survival. Antibodies reduced infectivity or virulence of the inoculum, allowing the host to eliminate the infection by supposedly different mechanisms.

In two reports of complete neutralization of infective inocula, the serum, however, was raised in hosts which were allogeneic (Golenser, Spira and Zuckerman 1975) or xenogeneic (Cox 1969) to the donors of the infected erythrocytes, and neutralization occurred only in vivo but not in vitro. It appeared that the heterologous antiserum contained anti mouse-erythrocyte opsonin, but was not directely parasiticidal. Phagocytosis of the infected mouse erythrocytes was therefore induced in the peritoneum, but parasites directly introduced into the blood stream by-passed the phagocytic peritoneal barrier, and they were capable of invading clean red cells in the circulation i.e. not coated with opsonin (Zuckerman 1970; 1977). Thus, although serum transfer has been shown to abrogate an infective challenge under certain experimental conditions, some doubt remains regarding the extent of involvement of antibody in the immune response of rodents to an ongoing infection.

Mechanisms by which antibodies may effect protection include opsonization (P. knowlesi, Brown 1971), schizont red cell agglutination (Brown, Brown and Hills 1968), inhibition of merozoites to invade red blood cells (Cohen and Butcher 1970; Butcher, Mitchell and Cohen 1973). In our lethal P. berghei - BALB/c model the role of antibodies themselve is minor. A number of considerations support this view. First, additional supply of hyperimmune serum to infected mice does not affect the parasitemia. Secondly, the infectivity of P. berghei parasites "preserved" in vitro in hyperimmune serum seems to be even better than after incubation in normal serum (Jerusalem 1966). Thirdly, the course of parasitemia in infected athymic mice (nu/nu) is not significantly different from that in normal mice of the same strain.

One should be careful, however, in interpretating these phenomena obtained in athymic mice. P. yoelii infection is self-limiting in normal mice and induces a moderate parasitemia. In athymic mice, however, the parasitemia and mortality is strongly increased (Weinbaum, Evans and Tigelaar 1976). The opposed results (P. berghei - P. yoelii) suggest that different mechanisms or differences in host immunological reactivity underlie the causes of death.

The problem of the quantity of antibodies in acutely infected mice is complicated by the existence of different types of antibodies as suggested previously (Jerusalem, Weiss and Poels 1971). Competition of distinct types of antibodies (e.g. enhancing anti-

bodies) for the same parasites might prevent a humoral and/or cell mediated cytotoxic killing of the parasites, and favour their survival.

Beside the quantity and quality of the antiplasmodial antibodies one should also consider the practical aspects of an exclusive humoral response in eliminating an intracellular parasite. The antibodies appear to react with circulating soluble antigens, free parasites and reticulocytes with multiple infection. They do not coat single infected reticulocytes or infected oxyphilic erythrocytes (Poels, van Niekerk, Franken and van Elven 1977). This means that a large proportion of the parasites escapes the action of antibodies when 1) the parasites are intracellular; 2) the antibodies can be blocked partially by released soluble antigens (or haptens). It is obvious that in the P. berghei - mouse model the antibodies themselves do not offer any real protection to the host.

A humoral specific immunity conprises B lymphocytes, complement and polymorphonuclear (PMN) leucocytes as the principal combatants. The effectivity of the humoral elimination system can be severely reduced by a deficiency of PMN leucocytes (absence or functional failure) in acutely infected mice. There is no indication that PMN cells play any role in P. berghei resistence. Unpublished observations in vitro and in vivo with rabbit or guinea pig complement failed to demonstrate any benifical effect on the infectivity and parasitemia respectively, as was also reported by Diggs, Shin, Briggs, Laudenslayer and Weber (1972).

II. How is the parasite eliminated in immune animals

An efficient effector mechanism will comprise the killing of intracellular as well as extracellular parasites. The possibility of killing (or inhibition of growth) of intraerythrocytic P. berghei parasites is suggested by an unpublished observations. When a hyperimmune mouse is injected intravenously with $5x10^8$ parasitized reticulocytes the parasites can be seen to degenerate and desintegrate within the reticulocytes during the subsequent days rather than in phagocytes. The intracellular killing or inhibition of growth is beyond the range of antibodies.

A second indication on what type of effector mechanism might be involved, is provided by the reported transfer experiments with immune spleen cells either to severely infected mice or to noninfected mice, yielding protection in the latter group (Poels, van Niekerk, Franken and van Elven 1977).

Both experiments are suggestive of the involvement of a cell-mediated immune reaction (CMI).

A cell-mediated specific immunity comprises T lymphocytes, lymphokines and mononuclear phagocytes. In the following paragraphs several types of cell-mediated immune reactions are discussed within the scope of possible defence mechanisms against malaria.

Cytotoxic T cells. The activated T lymphocytes themselves can be autonomous killer cells with a high lytic capacity without any requirement for antibodies, B lymphocytes or macrophages,

as has been convincingly demonstrated in allogeneic models in vitro (Lohman-Matthes and Fischer 1973; Henney 1977). Lysis is dependent on intimate association between effector and target cell. Lysis of target tumor cells rapidly occurs in vitro (less than 1 hour), and each effector cell can kill more than one target cell.

Immune lymphocytes may bring about cytolysis of certain target cells in a second way. Lymphocytes in response to specific antigens or in response to mitogens, release a soluble factor(s) (lymphotoxins) that can directly kill tumor cells (Granger and Kolb 1968; Henney 1973; 1977). The lymphotoxines themselves, although induced specifically by antigens, act in an unspecific manner and can damage other structures than target cells.

Cytotoxic macrophages. There are two pathways by which macrophages can be primed to become cytolitically active. One of these, defined as an "arming" process, may be due to the adsorption of cytophilic antibody onto the macrophage surface or to an acquisition of T cell factor. The "armed" macrophage reacts with the specific antigen (target cell). This specific reaction "activates" the macrophage, but the activated macrophages kills nonspecifically. Intimate contact between macrophage and target cell is required. This type of reaction may be considered as a potent helper and amplification mechanism of T cell cytotoxicity, particularly when it is taken into account that macrophages can be rendered cytotoxic repeatedly (Evans, Cox and Alexander 1973; Lohman-Matthes and Fischer 1973).

The other pathway by which macrophages can be rendered cytotoxic has been termed "activation" and will be discussed later as the reaction is nonspecific to an antigen.

Cytotoxic K cells. (Antibody-dependent cell-mediated cytotoxicity). The target cells are coated with antibodies. The effector cells bear receptors for the Fc fragment of IgG molecules enabling an interaction with the coated target cells. The effector cells are a heterogeneous group, but for a given target cell type one effector cell type appears to predominate. The effector cells are present in normal (nonimmunized) individuals. Very small amounts of antibody support cytolysis (Henney 1977). In contrast to cytotoxic T cells the effector cell in this reaction is functionally inactivated after interaction with an antibody coated target cell.

If one or more of these effector mechanisms were operational in P. berghei infected mice, a direct contact between effector cell and target cell occurring in small capillaries and lymphatic organs would be of prime importance. A prerequisite, however, would be that the infected parasitized red blood cells expose some plasmodial antigens in or at their surface in order to be recognized by the effector cells. This does not occur in all parasitized erythrocytes (Poels, van Niekerk, Franken and van Elven 1977).

The presence of soluble parasitic antigens and antigen/antibody complexes in infected mice would impair or inhibit such an effector mechanism in contrast to the situation in hyperimmune mice. A contribution of lymphotoxins to the eradication of the parasites

can only be expected if the lymphocytes, producing the toxins, are in the close environment of the parasitized cells as the effect will quickly be "diluted" in the circulation (Henney 1977).

Experimental studies which can provide sufficient grounds for concluding that a specific cell-mediated effector mechanism is involved in the defence against malaria are still lacking. It has been shown that transferred lymphoid cells are able to confer protection in rats (Philips 1970; Stechschulte 1969) and mice (Kasper and Alger 1973; Poels, van Niekerk, Franken and van Elven 1977). Increased susceptibility results from T cell deprivation in rats (Brown, Allison and Taylor 1968; Spira, Silverman and Gaines 1970) and mice (Barker and Powers 1971; Clark and Allison 1974). These data suggest an important role for T cell immunity in overcoming malaria infections. Direct killing, however, of parasitized erythrocytes by sensitized lymphocytes has been unsuccesful (Philips, Wolstencroft, Brown, Brown and Dumonde 1970; Weinbaum, Evans and Tigelaar 1976).

More evidence for the involvement of a cell-mediated cytotoxic reaction has been provided by Coleman, Rencricca, Stout, Brisette and Smith (1975) who showed that splenic cells effected *in vitro* specific lysis of ^{51}Cr-labelled erythrocytes from parasitized rodents. One effector mechanism involves splenic macrophages from normal or immune animals, which are increasingly cytotoxic to target cells in the presence of antibodies. A second effector

system concerns nylon purified immune spleen cells. Antibodies appear to enhance cell-mediated lysis. Their results, however could not be reproduced by others (Cohen, personal communication).

The cooperative action of antibodies in vivo was also demonstrated by Weinbaum, Evans and Tigelaar (1976) using B and/or T cells suppressed mice. Neither antibodies nor sensitized T-lymphocytes alone appeared sufficient to eliminate the parasite P. yoeli in mice, but the cooperation of both systems was required. The precise effector mechanism accomplishing complete elimination of parasitemia is unknown.

Nonspecific macrophages. Nonspecifically activated macrophages appear to contribute to the defence mechanism against malaria. Malaria infected mice pretreated with Corynebacterium parvum (Nussenzweig 1967) or Bacillus Calmette-Guérin (Clark, Allison and Cox 1976) showed decreased parasitemia and mortality. These agents have been shown to be potent stimuli to macrophage activation (Ratzan, Musher, Keusch and Weinstein 1972; Simon and Sheagren 1971; O'Neill, Henderson and White 1973), probably resulting from mediator production by sensitized T lymphocytes (Mackaness 1969; Krahenbuhl, Rosenberg and Remmington 1973). Once activated these macrophages enhanced nonspecifically phagocytosis and microbial killing (Simon and Sheagren 1971; Adlam, Broughton and Scott 1972). Although it has been suggested that BCG infection causes T cells or macrophages to produce factors that affect parasites within erythrocytes (Clark, Allison and Cox

1976), the presence or absence of antibodies was not examined nor was the possibility of B cell mediator production ruled out (Weinbaum, Evans and Tigelaar 1976).

Preliminary experiments with BCG and Freund adjuvants in our P. berghei - BALB/c mouse model, however, did not provide evidence for any beneficial effect on parasitemia or mortality.

Another (nonspecific) contribution of macrophages to the defence mechanism against malaria is the erythrophagocytosis in the lymphoid-macrophage system (reviewed by Zuckerman 1970; 1977). The erythrophagocytosis is stimulated in malaria infected rats (Zuckerman 1970; 1977) and mice (Kretschmar and Jerusalem 1963). All types of circulating erythrocytes, parasitized and unparasitized, are engulfed in the spleen, a feature which was considered to be the major reason of excessive anemia. Although this defence is a first line defence it is unprobable that it plays a significant role in killing of intracellular parasites as observed when hyperimmune mice were challenged with P. berghei.

In summary, the requirements for an efficient defence mechanism include (1) the neutralization of free parasites to such an extent that the infectivity is inhibited either by antibodies and PMN cells, or by specifically or nonspecifically activated macrophages; (2) neutralization of intracellular parasites by cell-mediated reactions through (a) release of cytostatic factors by activated lymphocytes, (b) liberation of intracellular parasites by a cytolytic reaction followed by neutralization by

antibodies and/or phagocytic cells, (c) direct killing of intracellular parasites by cell-mediated reaction or by soluble mediators from activated lymphocytes. All those mechanisms require the integrated function of both humoral and cellular factors.

The inefficiency of antibodies in our P. berghei - BALB/c mice model and the possibility of protective transfer of immune spleen cells to noninfected mice underline the need for investigations into the role of cell-mediated immunity in malaria infection. In addition, the knowledge of the (cell-mediated) effector mechanism will provide information on how to present plasmodial antigens (vaccins) to the host in such a structural configuration that preferentially the cell-mediated immune reaction will be activated, without evoking an interfering (humoral) immune reaction.

Table 1. Characteristics of Plasmodium berghei infection in BALB/c mice.

	ACUTE INFECTION		CURED INFECTION (a)
	DAY 0 - 7	DAY 7 - 21	DAY 42
1. Parasitemia	low (2-10%)	high (50-60%)	cleared
- intracellular	oxyphilic erythrocytes	polychromatophilic erythrocytes	-
- free parasites	present	present	-
- protective antigens	absent	present	-
- soluble antigens	—— detectable in small quantities —— (infected nude mice however accumulation of soluble antigens)		
2. Anemia	weak	severe	no
- reticulocytosis	normal level (0-2%)	high level (50-60%)	no
3 Splenomegaly	no (weight 1x)	yes (weight 10 15x)	no (weight 2x)
- red pulp	normal	hyperplasia	normal
- white pulp	normal	severely reduced	stimulated
- polysome profile	normal	anemic type	normal
4. Thymus	"normal"	involution	normal
5. Immune responsiveness to SRBC	initial increased than gradually reduced	completely suppressed	"normal"
- induction or tolerance to SRBC	no	yes	no
6. Protective immunity	no	no	(sterile) immunity
7. Immunoglobulin level			
a) antiplasmodial	low	low	high
b) non-antiplasmodial	high	high	high
8. Immune complexes in plasma and kidney	present	present	present
- containing plasmodial antigens	yes	yes	no
- proteinuria	+	yes	no
- rheumatoid factor	no	no	no
9. An acute infection is effected neither by transferred immune spleen cells nor by immune serum.			
10. Immune spleen cells can transfer protective immunity to non-infected recipients.			

(a) Chloroquine therapy between day 14 and 21 post infection.

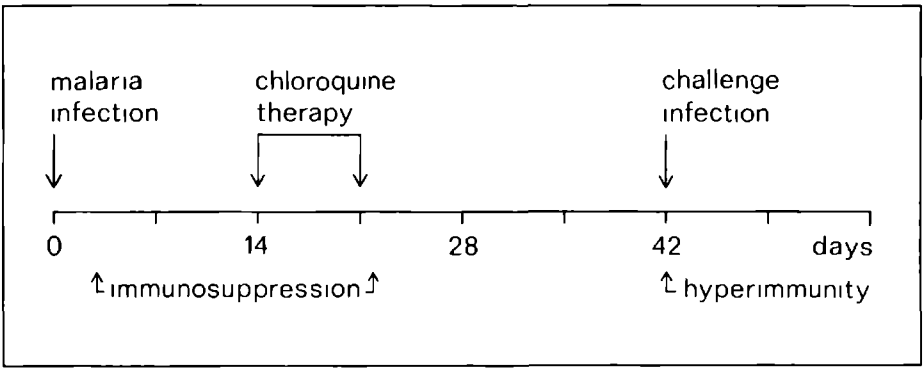
malaria
infection
chloroquine
therapy
challenge
infection
0
14
28
42
days
immunosuppression
hyperimmunity

Fig. 1.

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CHAPTER 8 SUMMARY

SUMMARY

Although considerable progress in global malaria eradication has been made in the past years, a large reservoir of endemic malaria remains over most of the tropics. The increasing emergence of drug-resistant strains of human plasmodia and of insecticide-resistant strains of mosquitoes, as well as difficulties of social-economic order, make it clear that malaria will be with us for quite some time, and that alternative approaches to malaria control will be necessary.

Although the final goal of malaria research might be the development of a malaria vaccine, an alternative approach was thought to be the use of "immunogenic" ("immune" or "informational") RNA which could possibly be instrumental in evoking a rapid antimalaria response. Transfer experiments with "immune" RNA or RNP particles require methods for isolation of original polysome structures and harvesting of adequate amounts of biologically active material. Therefore, the extent to which the polyribosome content in splenic extracts is affected by the level of endogenous ribonuclease activity, was investigated under ionic conditions optimal for preservation of polyribosomes (Poels 1976). The proportion of polyribosomes isolated from murine spleens on sucrose gradients was found to be affected by the balance between ribonuclease and its inhibitor, as well as the balance between initiation and translation process in protein synthesis. The distribution pattern of polyribosimes in sucrose gradients, thus, reflects in a way the physiological state of the cells.

Analysis of the splenic polysome profile during the course of a lethal Plasmodium berghei infection in Swiss mice (Poels 1977) revealed that the

onset of polyribosome formation coincides with the first peak of parasitemia and reflects the stimulated proliferation of erythropoietic tissue in the spleens rather than a proliferation of plasma cells constituting only a minor part in the malarious spleen.

Transfer of splenic ribonucleoprotein particles (or RNA), obtained from mice infected with or immunized to Plasmodium berghei, to normal recipients subsequently challenged, did not affect the parasitemia, but the number of animals succumbing in the period of the first peak mortality was either enhanced or reduced compared to controls, depending on the size of the inoculum (Jerusalem, Weiss and Poels 1971). Protective immunity, however, could not be transferred by RNP or RNA fractions. These negative results together with the inability to repeat RNA-induction experiments reported in the literature, and the limited understanding of the immune response of the host to the parasite made it imperative to characterize the host immune response to Plasmodium berghei infection in mice more precisely.

Three aspects have been studied in the Plasmodium berghei-mouse model.

Immune responsiveness. (Poels and van Niekerk 1977)

Plasmodium berghei infection can exert an adjuvant-like effect on the formation of Plaque Forming Cells when mice are infected one to three days after priming with Sheep Red Blood Cell (SRBC), stimulating proliferating SRBC-primed lymphocytes, but not resting SRBC-memory cells. The second period of infection (day 10-30) is characterized by a severe thymic involution, (T cell depletion), splenomegaly, complete suppression of the immune responsiveness to SRBC, and the inducibility of tolerance to SRBC. During the period of immunosuppression considerably elevated

levels of nonspecific antibodies, predominantly IgG are produced, while the level of thymus dependent IgA production is reduced. Antibodies with specificity to parasitic antigens are hardly detectable. When the infection is cured with chloroquine therapy between day 14 and 21 of infection, a gradual repopulation of the thymus is accompanied with a reconstruction of the splenic white pulp, a restore of the immune responsiveness to SRBC and an increase of serum IgA level. Serum IgG level remains high for a prolonged period and the antibodies are specific to plasmodial antigens. There is a possible relationship between immunosuppression and hyperimmunoglobulinemia.

Selective release of protective antigens (Poels, van Niekerk, Franken and van Elven 1977).

Although the immune responsiveness to heterologous erythrocytes is severely depressed in acutely infected mice, it does not imply *a priori* that the reactivity to plasmodial antigens is affected to the same extent. Secondly the building up of protective immunity to the parasite will be impaired only if the malarial antigens released before the period of immunosuppression are "nonprotective" antigens or "protective" but not available in sufficient quantities. Therefore, it was investigated which stage in the course of infection yielded "protective" antigens. Chloroquine therapy of an ongoing infection leads to the development of a (sterile) protective immunity only if the parasitemia is aborted not earlier than 14 days after infection when the parasites are present almost exclusively in reticulocytes. Active immunization under chloroquine conditions causes protective immunity when parasitized reticulocytes are used, in contrast to parasitized oxyphilic erythrocytes

obtained during the first seven days of an infection. These results suggest that "protective" antigens are released just in a period of severe immunosuppression thus preventing a spontaneous development of protective immunity without chloroquine therapy.

Immunofluorescence (LM) and immunoperoxydase (EM) studies revealed that only free parasites and those reticulocytes containing three of four large parasites are available for the action of antibodies. The character of the protective immunity was further explored by transfer experiments with immune serum and/or spleen cells. Although the first peak mortality in acutely infected mice can be prevented by administration of hyperimmune serum, the infection can not be aborted by immune serum and/or immune spleen cells. Nevertheless, in 60% of syngeneic mice protection has been transferred to noninfected recipients by giving them multiple injections of immune spleen cells during a period of four weeks prior to challenge.

Formation of immune complexes (Poels, van Niekerk, Pennings, Agterberg and van Elven 1977).

Although in the period of severe immunosuppression antiparasitic antibodies can hardly be detected, immune complexes containing immunoglobulins, complement and plasmodial antigens, are deposited in the glomeruli of kidneys causing proteinuria and ultrastructural alterations. Free circulating plasmodial antigens and immune complexes are present in malarious plasma, thus clearing up the plasma from specific antiplasmodial antibodies. After chloroquine therapy the plasmodial antigens disappear from the plasma and from the deposits in the kidney, while the fluorescence for IgG and complement in the glomeruli increases.

The hyperimmune plasma contains free antiplasmodial antibodies and immune complexes without plasmodial antigens. This second type of immune complexes is deposited in the glomeruli and causes comparable ultrastructural changes. Proteinuria, however, has not been observed.

The results have provided evidence that Plasmodium berghei infection in BALB/c mice causes a non-transient deposition of immune complexes of two different types: the first type in which plasmodial antigens can be demonstrated during an acute infection; a second type in which plasmodial antigens can not be demonstrated, and occurring in plasma of recovered hyperimmune mice.

CHAPTER 9 SAMENVATTING

SAMENVATTING

Hoewel er de laatste jaren aanzienlijke vorderingen zijn gemaakt bij de bestrijding van malaria, komt endemische malaria nog steeds veelvuldig voor in de meeste tropische landen. De malaria bestrijding wordt aanzienlijk bemoeilijkt enerzijds door de ontwikkeling van resistentie zowel van de parasieten tegen humaan toegepaste geneesmiddelen, als ook van de muggenpopulatie tegen insecticiden, anderzijds door sociaal-economische problemen in de ontwikkelingslanden. Derhalve is het wenselijk te zoeken naar alternatieve methoden om de malaria onder kontrole te krijgen.

Ofschoon het hoofddoel in de malariaresearch gericht zal zijn op het ontwikkelen van een vaccine, zou men het induceren van immuniteit door middel van "immunogeen" ("immuun" of "informatie"-) RNA kunnen beschouwen als een alternatieve mogelijkheid om een snelle antimalaria reaktie op te wekken. Om overdrachts- of inductie-experimenten met RNA- of ribonucleoprotein partikels te kunnen uitvoeren, moet men de beschikking hebben over isolatietechnieken waarbij de oorspronkelijke polysomenstruktuur behouden blijft en er toch een voldoende opbrengst wordt verkregen.
Daarom wordt onderzocht of in een milt-homogenaat het polysomengehalte samenhangt met de endogene ribonuclease aktiviteit, bij ionenconcentraties die optimaal zijn om de polysomenstruktuur intakt te laten (Poels 1976). Het gehalte aan polysomen met behulp van sucrose gradiënten uit muizenmilten geïsoleerd, blijkt afhankelijk te zijn van zowel het evenwicht tussen het enzym ribonuclease en

zijn remstof, als van het evenwicht tussen de initiatie en de translatie van de eiwitsynthese. Het verdelingspatroon van polyribosomen in sucrosegradienten weerspiegelt in zekere zin de fysiologische toestand van de cellen. Analyse van de miltpolysoomprofielen tijdens malaria infektie in Swiss muizen (Poels 1977) toont aan dat het begin van de vorming van polysomen samenvalt met de eerste piekparasitemie. Het polysomenprofiel is voornamelijk representatief voor het prolifererende erythropoietische weefsel in de milt en niet voor prolifererende plasmacellen, die overigens slechts in een relatief gering aantal voorkomen in de sterk vergrootte malaria milt.

Isoleert men polyribosomen of RNA uit de milten van muizen die geinfekteerd zijn met Plasmodium berghei of immuun zijn, en spuit men dit materiaal in normale muizen die vervolgens worden geinfekteerd, dan blijkt deze voorbehandeling geen invloed te hebben op het verloop van de parasitemie. Het aantal dieren dat dood gaat tijdens de eerste pieksterfte is, in vergelijking met de controles, òf verhoogd òf verlaagd, afhankelijk van de grootte van het inoculum (Jerusalem, Weiss en Poels 1971). Protektieve immuniteit kan echter niet worden geïnduceerd door middel van RNP of RNA frakties.

Deze negatieve resultaten, èn het onvermogen om inductie experimenten met RNA, als vermeld in de literatuur, te herhalen, alsmede het beperkte inzicht in de "immuun response" van de gastheer ten opzichte van de parasiet P. berghei, deden ons besluiten de immunologische reaktie tijdens de infektie nader in beschouwing te nemen.

Een drietal aspekten werden onderzocht in het P. berghei-muis model.

Immunologische reaktiviteit (Poels en van Niekerk 1977).

Plasmodium berghei infektie werkt als een soort adjuvans bij de vorming van Plaque Forming Cells indien de muizen eerst worden ingespoten met schapen erythrocyten (SRBC) en vervolgens binnen 1 tot 3 dagen met een inoculum van geparasiteerd bloed. Slechts de prolifererende gesensibiliseerde lymphocyten worden gestimuleerd, echter niet de rustende SRBC-memory lymphocyten.

De tweede periode van infektie (dag 10-30) wordt gekarakteriseerd door een ernstige involutie van de thymus (depletie van T cellen), splenomegalie, volledige onderdrukking van de immuunreaktie tegen SRBC, en de induceerbaarheid van tolerantie tegen SRBC. Tijdens de periode van immunosuppressie is het gehalte aan nietspecifieke immunoglobulinen, met name IgG, aanzienlijk verhoogd, terwijl het gehalte aan IgA, waarvan de produktie thymusafhankelijk is, sterkt daalt. Antiparasitaire antistoffen zijn tijdens de infektie nauwelijks aantoonbaar. Geneest men de infektie met een chloroquine behandeling tussen dag 14 en 21, dan raakt de thymus geleidelijk weer bevolkt, wordt de witte pulpa van de milt gerekonstrueerd, hersteld de immuunreaktie tegen SRBC zich en neemt het IgA gehalte in het serum weer toe. Het serum IgG gehalte blijft lange tijd verhoogd en de antistoffen blijken specifiek tegen parasitaire antigenen gericht. Mogelijk bestaat er een relatie tussen de immunosuppressie en de hyperimmunoglobulinemie.

Het selektief vrijkomen van "protektieve" antigenen (Poels, van Niekerk, Franken en van Elven 1977).

Ofschoon de immuunreaktie tegen SRBC ernstig gestoord is tijdens een akute infektie, impliceert dit niet a priori dat de immunologische reakties tegen plasmodiale antigenen in dezelfde mate geremd worden. De opbouw van een protektieve immuniteit tegen de parasiet zal bovendien alleen dan worden belemmerd indien de malaria antigenen, die vrijkomen voor de periode van immunosuppressie, òf geen "protektieve" antigenen zijn, òf niet in voldoende hoeveelheden vrijkomen om een protektieve immuniteit te kunnen induceren. Daarom werd onderzocht in welk stadium van de infektie antigenen vrijkomen die een protektieve immuniteit kunnen opwekken. Chloroquine behandeling tijdens een akute infektie blijkt alleen dan tot een (steriele) protektieve immuniteit te leiden, indien de parasitemie niet eerder wordt geëlimineerd dan op dag 14 van de infektie; op dit tijdstip zijn de parasieten bijna uitsluitend gehuisvest in reticulocyten. Aktieve immunisatie, onder chloroquine bescherming, induceert alleen dan protektieve immuniteit wanneer met geparasiteerde reticulocyten toedient, echter niet wanneer men niet geparasiteerde oxyfiele erythrocyten inspuit. Deze gegevens suggereren dat "protektieve" antigenen ter beschikking komen van de gastheer juist in een periode van ernstige immunosuppressie, zodat een spontane ontwikkeling van protektieve immuniteit in dit model niet mogelijk is zonder chloroquine behandeling.
Met behulp van immunofluorescentie (LM) en immunoperoxydase technieken (EM) is aangetoond, dat alleen de vrije parasieten en die reticulocyten die 3 à 4 grote parasieten bevatten blootgesteld kunnen worden aan de

werking van antistoffen.

De aard van de protektieve immuniteit werd verder onderzocht door het inspuiten van immuunserum en/of miltcellen in geinfekteerde dieren. Ofschoon de eerste pieksterfte tijdens een akute infektie kan worden voorkomen door toediening van hyperimmuunserum, kan de parasitemie niet worden bestreden met behulp van immuunserum en/of miltcellen. Niettemin, in 60% van de muizen is bescherming overgedragen aan niet geïnfekteerde normale muizen, nadat aan deze muizen een aantal injekties met immune miltcellen was toegediend gedurende 4 weken vóór een infektie met Plasmodium berghei.

Vorming van immuunkomplexen (Poels, van Niekerk, Pennings, Agterberg en van Elven 1977).

Tijdens de periode van immunosuppressie kunnen nauwelijks antiplasmodiale antistoffen worden aangetoond; toch blijken er immuunkomplexen in de nieren te worden afgezet, die een proteinurie veroorzaken en karakteristieke ultrastrukturele veranderingen in de glomeruli teweegbrengen. De immuunkomplexen in de nier bevatten immunoglobulinen, komplement en parasitaire antigenen.
In het plasma van geïnfekteerde dieren zijn cirkulerende antigenen aantoonbaar, die met de specifieke antistoffen komplexen vormen en aldus uit de bloedbaan verwijderd worden. Na behandeling met chloroquine verdwijnen de parasitaire antigenen zowel uit het bloed als uit de immuunkomplexen in de nieren. De intensiteit van de fluorescentie voor IgG en komplement in de glomeruli neemt echter toe.

Hyperimmuunplasma bevat zowel vrij cirkulerende antiplasmodiale antistoffen, als immuunkomplexen waarin geen parasitaire antigenen aantoonbaar zijn. Dit tweede type immuunkomplexen wordt eveneens afgezet in de glomeruli van de nieren. Ze veroorzaken weliswaar geen proteinurie, maar wel ultrastrukturele veranderingen in de glomeruli. Plasmodium berghei infektie in BALB/c muizen veroorzaakt dus geen tijdelijke maar een kontinue afzetting van twee typen immuunkomplexen in de glomeruli.

curriculum vitae:

Lambert G. Poels
Born 1943
Gymnasium-B 1956-1962
Student in Biology, Faculty of Science, Katholieke Universiteit, Nijmegen 1962-1969
At the Department of Cytology and Histology, Medical Faculty, Katholieke Universiteit, Nijmegen since 1970

STELLINGEN

I

De visie van Brown, Jarra en Hills dat een realistisch laboratoriummodel voor de immunologie van de malaria ook een chroniciteit van de infectie tot uitdrukking moet brengen, is niet realistisch.

Brown, K.N., Jarra, W., Hills, L.A. (1976)
Infection and Immunity, 14, 858-871.

II

Het is bedenkelijk om uitsluitend op grond van vergelijking van parasitemie en mortaliteit in malaria geïnfecteerde normale en thymusloze muizen conclusies te trekken omtrent de rol van T-cellen bij de immuniteit tegen malaria.

III

Experimentele immunisatie met sporozoïten leidt tot volledige bescherming tegen de door muggen overgebrachte malaria parasiet. Het ontbreken van dit effectieve verweer bij mensen in hyerendemische gebieden zou ondermeer toegeschreven kunnen worden aan malaria geïnduceerde immunosuppressie.

IV

Bij het gebruik van chemotherapeutica (b.v. stibogluconaat, sulfathiazol) om infectieziekten veroorzaakt door Leishmania en malaria te genezen en de ontwikkeling van een protectieve immuniteit te bevorderen, moet men rekening houden met een mogelijke interactie tussen de drugs en het immunologisch afweermechanisme.

Neal, R.A., Miles, R.A. (1977) Ann. Trop. Med. Parasitol., 71, 21-27.
Eling, W., Jerusalem, C. (1977) Z. Tropenmed. Parasitol. 28, 158-174.

V

Het is onbegrijpelijk dat Kabasawa en medewerkers de door hen onderzochte γ-cristallines van het rund niet aan "isoelectric focusing" hebben onderworpen.

Kabasawa, I., Tsunematsu, Y., Barber, G.W., Kinoshita, J.H. (1977) Exp. Eye Res. 24, 437-448.

VI

De morfologische veranderingen in milt en thymus van dystrofe muizen in de Bar Harbor 129/Rej stam, beschreven door De Kretser en Livett en Karmali en Horrobin, geven geen aanleiding tot de veronderstelling dat een autoimmuun proces betrokken is bij de spierdystrofie in deze stam.

De Kretser, T.A., Livett, B.G. (1976) Nature, 263, 682-684.
Karmali, R.A., Horrobin, D.F. (1976) Nature, 263, 684-685.

VII

Uit epidemiologisch onderzoek naar het voorkomen van anti-e en rheumafactor in hepatitis B patienten mag niet geconcludeerd worden dat beide factoren identiek zijn.

VIII

De Partij van de Arbeid zou haar steun aan het streven van de nederlandse vakbeweging om arbeid gelijkwaardig te stellen aan kapitaal symbolisch tot uitdrukking kunnen brengen in haar naamgeving.

IX

Men kan zich niet aan de indruk onttrekken dat tegenstanders van "genetic engineering" experimenten meer gemotiveerd worden door de vrees dat de mens in de toekomst genetisch gemanipuleerd kan worden dan door het directe gevaar dat genetisch manipuleren in microörganismen kan hebben voor de volksgezondheid.

X

De beleidsplannen van staatssecretaris G. Klein kunnen de indruk wekken dat hij, bij zijn verzuchting dat het onderzoek aan de nederlandse universiteiten meer grensverleggend zou moeten zijn, voornamelijk gedacht heeft aan het stellen van grenzen aan onderzoekstijd ten gunste van onderwijstijd in plaats van het instellen van een "sabbatical year".

XI

De resultatenverwachting van het aanbevelen, c.q. het invoeren van verplicht, algemeen hygiënische maatregelen ter preventie van infectieziekten bij de mens is evenredig aan het niveau van ontwikkeling van deze mensen.

Nijmegen, 2 december 1977 Lambert G. Poels